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ASSESSING PLANT TYPE AND THE NUTRITIVE VALUE OF SILAGE MAIZE IN THE PERSPECTIVE OF THE BELGIAN OFFICIAL VARIETY TRIALS

Thesis submitted in fulfillment of the requirements for the degree of Doctor (PhD) in Applied Biological Sciences: Agricultural Sciences

Dutch translation of the title: Beoordeling van planttype en voederwaarde van kuilmaïs in het kader van de Belgische officiële rassenproeven

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Woord vooraf

Met trots leg ik mijn doctoraat neer met de titel "Assessing plant type and the nutritive value of silage maize in the perspective of the Belgian official variety trials". Ik heb de voorbije vier jaar tussen kuilmaïs gewerkt en geleefd. Ik kan dan ook het doctoreren het best vergelijken met het de teelt van kuilmaïs. De maïs was reeds gezaaid toen ik startte met mijn doctoraat. Het perceel was gekozen: het doctoraat was een samenwerking tussen de Universiteit Gent en het instituut voor landbouw en visserij onderzoek (ILVO) waarvoor mijn dank om mij het vertrouwen te geven om dit doctoraat te starten. Een goede bodemkwaliteit verzekerde voor een groot deel de slaagkansen van de teelt. Ik wil dan ook een oprecht woord van dank richten aan mijn promotoren Prof. dr. ir. Dirk Reheul, dr. ir. Joke Pannecoucque, dr. ir. Johan De Boever voor de intensieve begeleiding, suggesties, vele raadgevingen en het aandachtig nalezen en corrigeren van deze tekst.

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Eens de plant het juiste drogestof gehalte bereikt, is de tijd aangebroken om te oogsten. De oogst en het kuilproces transformeren een plant tot veevoeder. Dit onderstreept de belangrijkheid van een multidisciplinaire samenwerking. Daarom dankuwel dr. ir. Johan De Boever (ILVO eenheid dier), Prof. dr. ir. Geert Haesaert (vakgroep toegepaste biowetenschappen), Prof. dr. ir. Kathy Steppe (vakgroep plant ecologie), Prof. dr. ir. Marie-Christine Van Labeke (laboratorium *in vitro* biologie en tuinbouw) en Prof. dr. ir. Veerle Fievez (vakgroep dierlijke productie) voor alle ondersteuning. Verder wil ik ook alle thesisstudenten bedanken: Andy, Astrid, Oscar, Vincent, Leen, Brent en Simon.

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Het doctoraat is nu een stabiel product, klaar om te voederen aan het grote publiek. Dankuwel aan u, de lezer, voor de interesse.

Summary

The incentive of the research reported in this PhD manuscript was a long lasting debate among the maize breeding companies and the conductors of the official Belgian variety trials. This PhD research mainly focused on the nutritive value of silage maize and intended to assess the currently used methods in variety testing and, if necessary, to propose alternative scientifically underpinned new methods.

Our research topics were:
(1) maize energy source: starch versus cell wall;
(2) variation in maize varieties: plant types and nutritive value;
(3) harvest window;
(4) the effect of ensiling on the nutritive value of maize;
(5) the effect of drought on the nutritive value of maize.

(1) The quality parameter cell wall digestibility (expressed as NDF digestibility (NDFD)) was investigated using animal trials and *in vitro* techniques. The animal trials revealed that energy type of maize silage (high starch or high NDFD) did not affect milk production, provided organic matter digestibility (OMD) is similar. Furthermore, we concluded that *in vitro* incubation with rumen fluid can be used as a more convenient, rapid and cheaper alternative for the *in situ* nylon bag technique to rank maize silages according to NDFD. The *in vitro* incubation with rumen fluid for 48 h continues to be the best practice for *in vitro* NDFD determination, although many modifications have been suggested. These modifications were introduced to either improve the precision (repeatability and reproducibility) or to save labour. However, the methods we tested, an enzymatic approach and the Daisy^{II} technique, could not meet these expectations. Ultimately, calculating NDFD based on starch concentration and OMD suffices to accurately rank NDFD.

(2) Throughout this thesis, we used eight forage maize varieties differing in earliness, energy source (starch or cell walls) and stay-green (SG) trait. The choice of these varieties was based on information given by the breeding companies. A complete comparison of the eight varieties was made after three years of field trials at three sites in Flanders. Physiological measurements, such as photosynthetic capacity and chlorophyll concentrations, were used to qualify and quantify the SG trait. We identified a normal plant type and a SG plant type, the latter having a delayed senescence. The main conclusion was that the SG trait provokes a shift in dry matter (DM) and nitrogen (N) partition between the stover and the ear. Compared to normal varieties, SG varieties had a smaller ear and a larger stover fraction; ears contained less, stover more N. The nutritive value was studied during maturation (from 25 to 40% DM concentration). Harvesting silage maize at a high DM concentration maximized DM yield, starch accumulation and OMD, whereas neutral detergent fibre (NDF) and NDFD decreased. The SG trait had a positive effect on maize nutritive value. During the whole grain-filling period, SG varieties had a greater starch concentration, greater OMD, smaller NDF and greater NDFD in the whole-crop and stover. Differences in nutritive value between the plant types were most pronounced in the stem.

(3) Silage maize variety testing systems usually evaluate new varieties by harvesting all varieties on a single harvest date. Under this testing system, only few varieties are harvested at the physiological stage where they theoretically show their optimal performance. The optimal harvest date was calculated as the date where whole-crop DM yield, ear DM yield (or starch concentration) and OMD were maximal. The variety rank at the optimal harvest date was compared with the variety rank at any studied single harvest date. Harvest dates where the variety rank was not statistically different from the rank at the optimal harvest date were pooled in a 'harvest window'. The concept of a harvest window was tested on a historical dataset. Our harvest-date trial with eight maize varieties, harvested at six harvest dates (from 25 to 40% DM concentration) in Merelbeke, Bassevelde and Ravels in 2013-2015 was used to validate the first results. Based on both datasets, the harvest window comprised a flexible harvest period of about 14 days. It was therefore concluded that applying a single harvest date is scientifically justified for the ranking of silage maize varieties in Belgium, when the whole-crop DM concentration of all varieties in the trial varied between 28 and 40% with a maximum difference of 7% between all compared varieties.

(4) Forage maize is nearly exclusively fed as a silage. We studied the effect of ensiling on the nutritive value of eight silage maize varieties throughout maturation. At six harvest dates, fresh samples were taken and half of the sampled material was ensiled in laboratory silos for 20 weeks. An optimal harvest date was calculated for both fresh maize and maize silage. A harvest window was defined as a set of harvest dates for which the variety ranking of the fresh maize corresponds with the variety ranking at the optimal harvest date calculated from the maize silage. Eventually, harvesting the silage maize at a DM concentration of 32-35% guaranteed an optimal harvest date. Based on the results of eight varieties, reporting variety ranks without going through the ensiling process continues to be a scientifically justified practice in the Belgian official variety trials. Varieties with a superior fresh quality keep their leading position after ensiling, but variety differences become smaller after ensiling.

(5) The nutritive value of maize is affected by drought stress. A field trial was conducted in 2013 and 2015 with half of the field irrigated at the crucial growth stages and the other half exposed to drought stress. Drought stress occurred during the flowering period, in which maize is generally considered most susceptible. Results of the effect of irrigation on maize nutritive value were contradictory between experimental years although the same experimental design was used.

Samenvatting

Het onderzoek in dit doctoraat is ontstaan uit het langdurige debat tussen de maïsveredelingsbedrijven en de mensen van de officiële Belgische rassenproeven. Dit doctoraatsonderzoek richtte zich vooral op de voederwaarde van kuilmaïs en beoordeelde de methoden die momenteel gebruikt worden om de rassen te testen. Indien nodig worden alternatieve, wetenschappelijk onderbouwde methoden voorgesteld.

De onderzoeksonderwerpen waren:
(1) energiebron van maïs: zetmeel of celwand;
(2) variatie in maïsrassen: plant types en voederwaarde;
(3) oogstvenster;
(4) het effect van inkuilen op de voederwaarde van kuilmaïs;
(5) het effect van droogte op de voederwaarde van kuilmaïs.

(1) De kwaliteitsparameter celwandverteerbaarheid, uitgedrukt als NDF verteerbaarheid, werd bestudeerd door gebruik te maken van dierproeven en *in vitro* technieken. Uit de dierproeven bleek dat de energiebron (zetmeel of celwand) van de kuilmaïs geen effect had op de melkproductie op voorwaarde dat de organische stof verteerbaarheid gelijk was tussen de rassen. Bovendien concludeerden we dat de *in vitro* incubatie met pensvocht gebruikt kan worden als een gemakkelijk, vlug en goedkoper alternatief voor de *in situ* nylonzakjes techniek om maïsrassen te rangschikken op hun celwandverteerbaarheid. De standaard *in vitro* incubatie met pensvocht voor 48 u blijft de best geschikte methode om de celwandverteerbaarheid te bepalen *in vitro*, ook al werden veel aanpassingen aan de methode voorgesteld in de literatuur. Deze aanpassingen werden voorgesteld om de precisie (herhaalbaarheid en reproduceerbaarheid) te verbeteren of om werk te besparen. Maar, de methoden die wij testten (een enzymatische benadering en de Daisy^{II} techniek) konden deze verwachtingen niet invullen. Uiteindelijk volstaat een berekening van de celwandverteerbaarheid op basis van het zetmeelgehalte en de organische stof verteerbaarheid om de celwandverteerbaarheid accuraat te voorspellen.

(2) Doorheen dit doctoraat gebruiken we acht kuilmaïsrassen die verschillen in rijpheid, energiebron (zetmeel of celwand) en 'stay-green (SG)' eigenschap. De keuze van deze rassen was gebaseerd op informatie verkregen bij de veredelingsbedrijven. Pas na het aanleggen van veldproeven gedurende drie jaar op drie locaties in Vlaanderen konden de rassen volledig vergeleken worden. Fysiologische metingen zoals fotosynthesecapaciteit en chlorofylconcentratie werden gebruikt om de SG eigenschap te kwalificeren en te kwantificeren. We vonden een normaal planttype en een SG planttype waarbij de laatstgenoemde een vertraagde veroudering vertoonde. De conclusie hierbij was dat de SG eigenschap vooral een verandering teweeg brengt van droge stof en stikstof accumulatie tussen de restplant en de kolf. Vergeleken met normale rassen hadden SG rassen een kleinere kolffractie en een grotere restplantfractie; kolven hadden een lager, restplant een hogere N concentratie. Kwaliteitsparameters (ruw eiwitgehalte, zetmeelgehalte, organische stof verteerbaarheid, celwandfractie en celwandverteerbaarheid) werden bestudeerd tijdens de afrijping (25 tot 40% drogestofgehalte). Kuilmaïs oogsten bij een hoog drogestofgehalte zorgde voor een maximale drogestofopbrengst, zetmeelgehalte en organische stof verteerbaarheid, terwijl de celwandfractie en celwandverteerbaarheid daalde.

De SG eigenschap had een positief effect op de kwaliteit van maïs. Tijdens de volledige graanvullingsperiode hadden de SG rassen een grotere zetmeelconcentratie, een grotere verteerbaarheid van de organische stof, een kleinere celwandfractie en een grotere celwandverteerbaarheid, zowel in de totale plant als in de restplant. Verschillen in voederwaarde tussen de planttypes waren het meest uitgesproken in de stengel.

(3) Rassenproeven met kuilmaïs evalueren nieuwe rassen door alle rassen te oogsten op éénzelfde oogstdatum. Dit systeem zorgt ervoor dat slechts enkele rassen geoogst worden op het moment waarop ze hun optimale prestatie bereiken. Het optimale oogsttijdstip werd berekend als het moment waarop de totale opbrengst, kolfopbrengst (of zetmeelgehalte) en verteerbaarheid van de organische stof maximaal waren. De rassenvolgorde op dit optimale oogsttijdstip werd vergeleken met de rassenvolgorde op elk bestudeerd oogsttijdstip. Tijdstippen waarbij de rassenvolgorde niet statistisch verschilde van de rassenvolgorde op het optimale oogsttijdstip werden samengebracht onder een 'oogstvenster'. Het concept van een oogstvenster werd getest op een historisch beschikbare dataset. De veldproef met acht rassen geoogst op zes oogsttijdstippen in Merelbeke, Bassevelde en Ravels in 2013-2015 werd gebruikt om deze eerste resultaten te valideren. Gebaseerd op deze twee datasets concludeerden we dat het oogstvenster een flexibele periode van 14 dagen omvat. Deze conclusie bewijst dat een vast oogsttijdstip voor alle rassen wetenschappelijk correct is om een rangschikking te maken van alle kuilmaïsrassen, indien het drogestofgehalte tussen 28 en 40% gelegen is met een maximaal verschil van 7% tussen alle vergeleken rassen.

(4) Kuilmaïs wordt bijna uitsluitend gevoederd als kuilvoeder. Wij bestudeerden het effect van inkuilen op de voederwaarde van acht kuilmaïsrassen doorheen de afrijping. Op zes oogsttijdstippen werd de helft van het materiaal ingekuild in microkuilen voor een duur van 20 weken. Een optimaal oogsttijdstip is berekend voor zowel vers als ingekuild materiaal. Een oogstvenster is gedefinieerd als een set van oogsttijdstippen waarbij de rassenvolgorde van het vers materiaal overeenkomt met de rassenvolgorde op het optimale tijdstip berekend met het ingekuild materiaal. Uiteindelijk werd het optimaal oogsttijdstip bereikt als men oogst bij een drogestofgehalte van 32-35%. We concludeerden dat het rapporteren van de rassenvolgorde op basis van vers materiaal wetenschappelijk correct is zonder het volledige kuilproces te doorlopen. Rassen met een superieure kwaliteit behouden hun leiderspositie na inkuilen, maar rasverschillen worden kleiner na inkuilen.

(5) De voederwaarde van kuilmaïs wordt beïnvloed door droogtestress. Een veldproef werd uitgevoerd in 2013 en 2015 waarbij de éne helft van het veld werd geïrrigeerd tijdens de cruciale groeifasen en de andere helft van het veld werd blootgesteld aan droogtestress. Droogtestress kwam voor tijdens de bloei wanneer de plant het meest gevoelig is. Echter, het effect van irrigeren op de kwaliteit van kuilmaïs was tegenstrijdig tussen de jaren ook al werd dezelfde proefopzet gebruikt.

List of Abbreviations

ADF	acid detergent fibre
ADL	acid detergent lignin
C	carbon
CP	crude protein
DM	dry matter
DMI	dry matter intake
DVE	true protein digested in the intestines
H_{opt}	optimal harvest date
MOU	measuring Ontario Units
N	nitrogen
NDF	neutral detergent fibre
NDFD	NDF digestibility
NE_L	net energy lactation
NIRs	near-infrared spectroscopy
OEB	degraded protein balance
OM	organic matter
OMD	organic matter digestibility
OU	Ontario Units
P_{sat}	photosynthetic capacity
SG	stay-green

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1 Introduction

1.1 Forage maize

1.1.1 General introduction

Maize (*Zea mays* L.) is one of the world's three major cereal crops (along with rice and wheat). In Europe, forage maize is an important component of the ration on most dairy farms for the last 40 years. The area used for forage maize cultivation has expanded rapidly in northern Europe after the release of the first early hybrids (Barrière *et al.*, 1997), but stagnated over the last decade. Currently, maize is the second arable crop in Belgium in terms of area, after grassland. In 2015, about 173 000 ha was cultivated with silage maize (Official statistics Belgium). This is 13% of the total cultivated area. In Germany, the maize growing area almost doubled between 2004 and 2014 due to the increased demand for maize for biogas production (Laidig *et al.*, 2014). Because silage maize can be harvested before grain maturity, the temperature requirements of forage maize are smaller than those of grain maize. Forage maize can be grown in areas with a mean seasonal temperature of 13.5 °C and above. Optimization of crop management (e.g. the use of early-maturing varieties and plastic mulch) has permitted the commercial production of forage maize in some climatically marginal locations (Lynch *et al.*, 2012).

Maize yields have steadily risen over the last century due to both genetic and management changes. Maize yield potential is defined as the maximum yield obtained by a genotype developed in an adapted environment, with non-limiting water and nutrient resources, under no pressure of pests and diseases, using the best management practices for the specific variety, weather and soil conditions (Ciampitti & Vyn, 2012). The increase in maize yield over the past 50 years may be attributed roughly 50% to genetic improvement (plant breeding) and 50% to improved management (Duvick, 2005). The equal contribution of plant breeding and management was also found in a UK study (Mackay *et al.*, 2011) in forage maize and sugar beets, whereas for cereals and oil seed rape at least 88% of the improvement in yield was attributed to plant breeding. As management improvements are becoming limited (use of nitrogen (N) and other chemicals are becoming more and more restricted), genetic improvement may be even more important as the means to increase yields over the next 50 years. Laidig *et al.* (2014) reported on yield trends in the period 1980-2010 using data from the official German variety trials. They split yield trends in a genetic and an agronomic component (Figure 1.1). While the genetic trend was continuously increasing with relative small year-to-year variability, the agronomic trend decreased with a large variation pattern. The declining agronomic trend was due to legal restrictions, climate change and management changes. The right part of Figure 1.1 shows that the declining trend was compensated by the genetic improvements. However, there is a large yield-gap between trials and farmers' fields as indicated in the right part of Figure 1.1 meaning that genetic progress is only partially transferred into practice. On-farm yields can range from roughly 20 to 80% of the potential yield due to management practices (Lobell *et al.*, 2009).

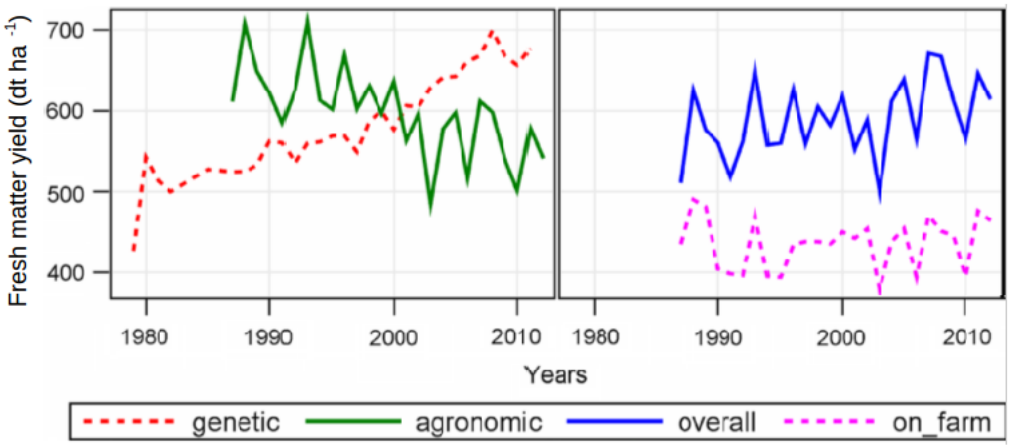


Figure 1.1: Fresh matter forage maize yield: genetic, agronomic and overall progress based on German VCU trials and compared to German average on-farm yields (Laidig *et al.*, 2014)

Further improvements in the yield of silage maize can be achieved by a more efficient use of radiation. Plants with a longer active photosynthetic capacity can potentially have greater yields. Indeed, prolonged photosynthetic activity in the leaves through delayed leaf senescence is one of several traits that have contributed to the increased potential of new hybrids (Tollenaar, 1991). Compared to older genotypes, senescence occurs later or at a slower rate in modern hybrids (Duvick, 2005). Furthermore, delayed senescence is associated with increased carbon (C) and N assimilation during the grain-filling period (Rajcan & Tollenaar, 1999) which resulted in a greater dry matter (DM) accumulation of newer vs. older hybrids (Valentinuz & Tollenaar, 2004) and enhanced DM partitioning to the grain. However, from a physiological point of view, plant nutrient uptake, assimilation and allocation are equally important for a balanced increase in source (photosynthesis) and sink (ear development) components. Genetic gains in maize yield may be accompanied by changes in other traits, either or not intentionally changed by the breeders. Duvick (2005) gives some examples: reduced ear height; upright leaf orientation; decreased tassel weight; increased leaf area index; leaf rolling as a drought response; delayed senescence; shorter anthesis-silking interval; longer period of grain-filling; increased kernel weight; decreased grain protein concentration. Other traits such as leaf number, heat units from planting to anthesis and harvest index have stayed unchanged over generations. Genetic improvement of maize yield is also related to increased stress tolerance to: high or low temperatures; drought or excessive soil moisture; low nutrient availability; increased population density and competition from weeds (Duvick, 2005).

Forage maize is frequently exposed to environmental abiotic and biotic stress during the growth season. Stress conditions may induce premature senescence of leaves, resulting in a shortage of assimilates and strongly affecting crop productivity (Gregerson *et al.*, 2013). On a global scale, water stress is the environmental factor that has the strongest negative impact on crop plant productivity (Chaves *et al.*, 2002). Periods of water stress can also occur in Belgium although rainfall is on average 800 mm per year (KMI, 2016). During the growth period of maize (from May to September), average rainfall in Belgium of 450 mm corresponds with the amount of water required by maize during its life cycle (Bänzinger *et al.*, 2000). However, due to the changing climate, periods of drought do and will occur more frequently, along with an increasing inter-annual variability (Ergon *et al.*, 2016). Water stress is normally associated with high temperature and high light stresses. Therefore, plant responses to drought stress are often

correlated with responses to the stresses associated with drought.

1.1.2 Nutritive value of forage maize

Yield and earliness were and still are the main objectives in maize breeding programmes, but more and more attention is paid to nutritive quality. Both vegetative and generative tissues are chopped and mixed within the maize silage. Therefore, the feeding value of maize silage is determined by the ear and the stover, providing energy in the form of starch and structural fibre respectively. As the stover is proportionately 0.35-0.45 of the total DM in maize plants, a considerable proportion of the nutritive value comes from cell wall material (Boon *et al.*, 2005). Therefore, an improvement in the stover digestibility, independent of the ear component, would present an opportunity to increase the nutritive value of the whole-crop. Another breeding strategy is to increase energy content by maximizing starch concentrations. However, a high concentration of fast degradable starch in the ration decreases rumen pH, which may impair the health of ruminants. Furthermore, cell wall digestibility in the rumen and digestion of starch in the intestines become limited with high amounts of starch (Dijkstra, 1993; Van Vuuren *et al.*, 2010).

Starch is almost completely digestible because carbohydrates that escape the rumen can be enzymatically digested in the small intestine. In the rumen, starch is fermented to volatile fatty acids that deliver energy to the animal and supplies ATP for microbial protein production. Starch digestion in the small intestine produces glucose as an end product that can be used more efficiently than volatile fatty acids by the animal (Nocek & Tamminga, 1991). The site of starch digestion (rumen or small intestine) depends on the composition (amylose/amylopectin ratio), endosperm type (dent or flint) and texture of the grains. These variables depend on the maize genotype, growing conditions, maturity stage at harvest, mechanical processing and preservation. The composition in starch varies between 20-30% amylose and 70-80% amylopectin. Because amylose is more resistant to enzymatic hydrolysis than amylopectin, the ruminal degradability of starch is negatively correlated with the proportion of amylose in the total starch (Khan *et al.*, 2014).

Forage maize digestibility is greatly affected by the concentration of NDF and NDF digestibility (NDFD). No correlation exists between NDF and NDFD (Barrière *et al.*, 2003), so a high nutritive value can be achieved by increasing NDFD of the stover. Feed rations for ruminants should contain sufficient NDF to maintain rumen function: chewing activity and rumen pH are closely related to the concentration of NDF. However, excess NDF may limit voluntary feed intake because of physical fill in the rumen. Feed intake can be improved with greater NDFD so NDF can disappear more rapidly from the rumen (Oba & Allen, 1999). Krämer-Schmid *et al.* (2016) found an improved daily milk yield of 82 g and daily weight gain of 12 g when NDFD increased with 0.01 units.

The energy content of feeds for ruminants is expressed in either metabolizable energy or net energy (De Boever & De Campeneere, 2015). Metabolizable energy is obtained by subtracting energy losses (from the rumen, faeces and urine) from the gross energy (heat of combustion). Net energy also takes into account the heat losses during metabolism. Metabolizable energy is mostly calculated from the digestible nutrients obtained by digestion trials with sheep. The net energy can be calculated from the contents and digestibility of crude protein, crude fibre, crude fat and other carbohydrates. A shortcoming of these systems is that they do not consider the amount and type of nutrients. Animal requirements can only be met when the exact energy and protein contents are known, consequently avoiding unnecessary losses from respiratory, faecal and urinary excretion into the environment (Dijkstra, 1993).

The reference method to determine rumen degradation characteristics of feedstuffs is the *in situ* incubation technique (De Boever *et al.*, 2002). However, digestibility experiments with animals are expensive, time-consuming and require large quantities of feed which make them unsuitable for routine feed evaluation. Laboratory *in vitro* methods using rumen fluids can accurately predict the nutritive value of forages, but they still need fistulated animals. De Boever *et al.* (1988) introduced an *in vitro* enzymatic method to determine organic matter digestibility (OMD) which is simpler to conduct and has a better reproducibility. Over the last 20 years, the fast near-infrared spectroscopy (NIRs) technology has been introduced in maize silage evaluation (De Boever *et al.*, 1997). NIRs characterizes the entire sample in terms of its absorption properties in the NIR region. Analysis of forage samples by NIRs offers the advantages of simplicity, speed of analysis, reduced chemical waste and more cost-effective prediction (De Boever *et al.*, 1997).

1.1.3 Plant types

Maize plant development can be divided into vegetative and reproductive stages (Ritchie *et al.*, 1997), but a more consistent and continuous descriptor of plant maturity is the whole-crop DM concentration. DM concentration can be used as a covariate for statistical purposes and is used in variety trials to rank the relative variety maturity (Johnson *et al.*, 1999). However, DM concentration is also influenced by the weather thereby causing some variation in DM concentration at specific maturity stages. The final stage of plant development involves a whole-plant senescence process. A developing leaf goes through three main phases: an expansion phase, a maturity phase and a senescence phase (Figure 1.2). The non-reversible senescence process, starting from the 'point of no return', eventually ends with the death of the leaf. Leaf senescence is characterized by a loss of chlorophyll, apparent to the eye as a loss of green color. The timing, rate and efficiency of the senescence process is complex and highly regulated (Gregerson *et al.*, 2013).

Senescence is not only a degenerative process because the N released from the senescent leaves is recycled in the plant to the benefit of developing seeds in the ear. As most of the N is associated to the activity of the photosynthetic apparatus, the onset and rate of leaf senescence is related to the balance between N demand by the ear and N supply during grain filling. Therefore, variations in senescence appearance can be explained by N abundance and distribution in the plant (Borrell *et al.*, 2001).

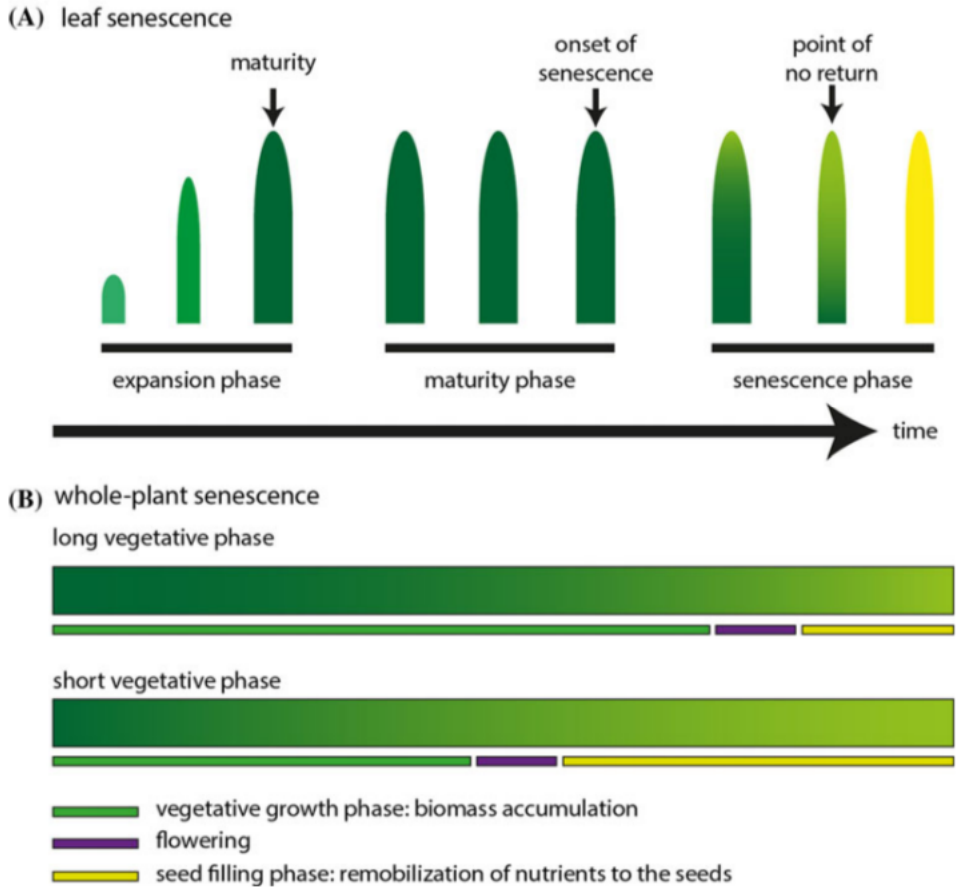


Figure 1.2: Phases of leaf and whole-crop development (Gregerson *et al.*, 2013)

The importance of N for maize growth is closely linked to its role in photosynthesis due to the impact on photosynthetic rates and because of the need for N during photosynthate translocation to the developing ear. N is a main constituent of endosperm storage protein and it is essential for the enzymatic processes during ear growth (Cazetta *et al.*, 1999). The N flux to the ear consists of two components: N uptake from the soil and N remobilization from vegetative tissues (Borrell *et al.*, 2001). As a C4 photosynthesis plant, maize has a low N reserve in the leaves. Consequently, the generative development of the maize plant is dependent on post-silking N uptake (Pommel *et al.*, 2006). Newer hybrids delay N mobilization from vegetative tissue, increasing the duration of leaf photosynthesis (Ciampitti & Vyn, 2012). Because of the inverse correlation between N uptake and N mobilization, recent hybrids depend more on N taken up by the roots. Indeed, recent hybrids had larger roots (Ning *et al.*, 2014) and a greater ratio of post-silking N uptake on total N in the ear compared to older ones (Rajcan & Tollenaar, 1999).

At the beginning of the growing season, N supply exceeds the crop demand when fertilizers are applied. As the season progresses, N depletion of the soil can occur because N is taken up by the plant. Depending on the timing of N stress, maize yield can be affected differently. Before flowering, N stress reduces leaf area development, photosynthesis rate, and the number of ear spikelets (potential grains). During grain-filling, N stress induces leaf senescence and reduces crop photosynthesis and kernel weight (Bänzinger *et al.*, 2000).

The stay-green (SG) term is used in studies with varieties showing delayed senescence in the field. However, delayed appearance of visual symptoms of leaf senescence does not guarantee a longer duration of photosynthesis. According to Thomas & Howarth (2000), different types of SG exist, with the two possible results that SG types are functional or cosmetic (Figure 1.3). Functional SG hybrids photosynthesise longer than normal hybrids owing to a delayed onset (Type A) and/or a slower decrease in photosynthetic capacity (Type B). Cosmetic SG hybrids do not photosynthesise longer but accumulate pigments in the leaves, giving the impression that there is a reduction of senescence (Type C), maintain the green color with leaf death via freezing, boiling or drying (Type D) or increase chlorophyll concentration resulting in a delay in yellowing of leaves (Type E).

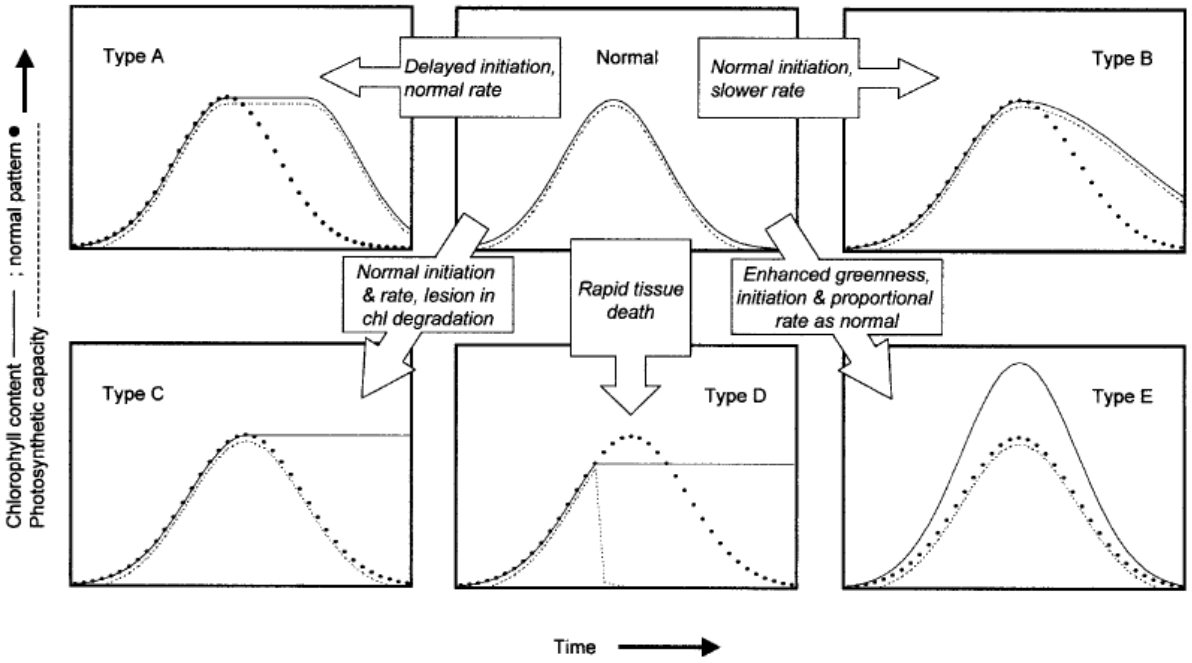


Figure 1.3: Five ways to stay-green (Thomas & Howarth, 2000)

By delaying senescence, SG varieties have the opportunity to intercept more solar radiation and the potential to accumulate more DM. However, a high DM yield can only be achieved when the strength of source (photosynthetic capacity) and sink (developing ear) are balanced. Whereas the grain yield for wheat is mainly limited by sink strength, the yield in maize depends on maintenance of high activity of the source (Gregerson *et al.*, 2013). Functional SG varieties have an increased source-sink ratio during grain filling. When the supply of assimilates exceeds the demand, the surplus of assimilates can be translocated to the roots, resulting in a greater post-flowering N uptake. But most of the surplus of assimilates is temporarily stored in the stover, which acts as a buffer, illustrated by Rajcan & Tollenaar (1999) providing that DM accumulated in the stover when assimilate supply is greater than the demand by the ear. The greater accumulation of DM in the stover was also associated with a greater lodging resistance in a study comparing old with new hybrids (Tollenaar, 1991). The SG trait has been associated with reduced plant lodging, increased disease and pest resistance and good plant health later in the season (Thomas & Smart, 1993).

The stage of physiological development at harvest is a major factor in determining the nutritive value of forage maize. Delayed senescence, due to the SG trait, is associated with an asynchronous maturation rate of the ear and stover. Consequently, the long used relationship between whole-crop DM concentration and maturity status no longer holds for SG varieties: the grain is at the optimal stage of maturity when the vegetative parts are relatively immature. Therefore, harvesting SG varieties at the traditional grain maturity stage can result in excess moisture concentrations in the silage. However, the theory that greater moisture concentrations of SG varieties may affect silage pH and stability was not supported by Arriola *et al.* (2012).

1.1.4 Silage process

Part of the success of forage maize in Belgium is the ease of preservation for feeding at a later date. Maize silage is an important part in ruminant feeding when grazing is restricted, but it also plays a significant role as a supplement during the grazing period. A good silage quality is ensured by a good silo management throughout the entire ensiling process, from filling to feedout (Wambacq *et al.*, 2016). The silage process consists of three phases (Kung, 1996): 1) the rapid removal of air, 2) the rapid production of lactic acid that results in a rapid drop in pH, and 3) continued exclusion of air from the silage during storage and feedout (Figure 1.4). The first step in the silage process is an aerobic phase with consumption of oxygen by green cells and aerobic organisms. The second phase of the silage process starts when all air is removed. An anaerobic maize silage is achieved by good compaction and fast sealing of the silo. The ease of compaction is closely related to the DM of the harvested material (McDonald *et al.*, 1991). Lactic acid bacteria convert water-soluble carbohydrates into lactic acid, responsible for decreasing the pH.

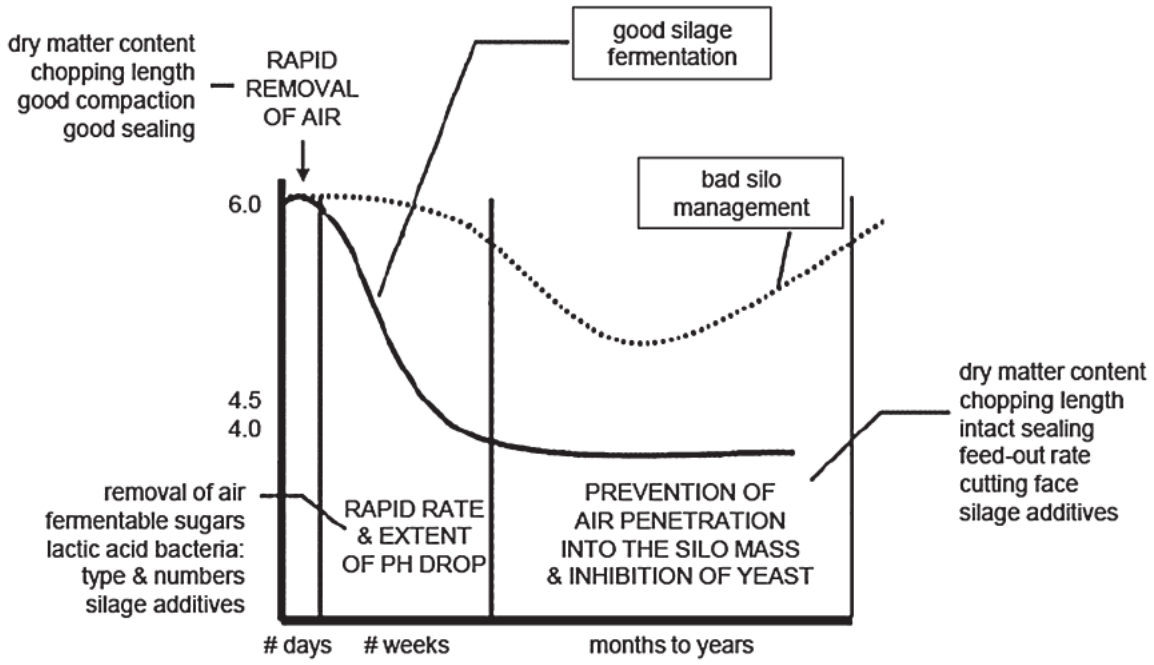


Figure 1.4: Evolution of pH during the three major events that make good silage, with indicators of factors that may affect the silage fermentation process (Kung, 1996)

The active metabolic processes in the silo cease after about 2 to 6 weeks of ensiling provided air is prevented from penetrating the silage, resulting in a stable phase of ensiling. The sealing should remain intact during the whole ensiling period and feedout can start after three months (Wambacq *et al.*, 2016).

Fermentation is a spontaneous process depending on DM concentration and carbohydrate availability. These factors interfere with the objective of stimulating and sustaining a low pH under anaerobic conditions to discourage the activities of plant enzymes and unwanted microorganisms. These microorganisms are inhibited at a pH below 4.5 when maize is harvested at a DM concentration of about 32% (Pahlow *et al.*, 2003). The quality of the silage process can be evaluated by measuring fermentation characteristics including pH, lactic, acetic, propionic and butyric acids, ammonia and ethanol (Table 1.1). Lactic acid should be the primary acid in good silage (at least 65 to 70% of the total silage acids). Clostridium bacteria ferment both carbohydrates and proteins, resulting in ammonia, ethanol and a mixture of organic acids (acetic, propionic, and butyric acids) (Pahlow *et al.*, 2003).

Table 1.1: Concentrations of fermentation end products in good maize silage (at 30-40% dry matter)(Kung & Shaver, 2001)

pH	3.7-4.2
Lactic acid (%)	4-7
Acetic acid (%)	1-3
Propionic acid (%)	<0.1
Butyric acid (%)	0
Ethanol (%)	1-3
Ammonia-N (% of CP)	5-7

CP=crude protein

Comparative, statistically sound studies of maize silage usually lean on laboratory mini-silos, mimicking the fermentation process in a way that is close to practical conditions. They have to be designed in a way that the entire content can be weighed, processed, and analysed accurately (Cherney *et al.*, 2004).

1.2 Variety trials

1.2.1 Regulations and procedure (based on Van Waes (2009))

This doctoral research is performed in the context of the Belgian official variety trials. Therefore, the procedure for maize variety testing in Belgium is explained below based on the publication of Van Waes (2009).

Variety research has been regulated at the European level via council directives for more than 45 years. Each member state has to translate the directive into its national and regional laws and produce a National List of varieties. Based on the member states' National Lists, the European Commission compiles the Common Catalogues of varieties. From the moment that a variety is registered on a National List, and consequently transferred into the Common Catalogue, it can be freely commercialised throughout the EU. Acceptance on a National List requires that every new variety undergoes official trials for DUS (Distinctness, Uniformity, Stability) and VCU (Value for Cultivation and Use), performed in accredited research institutes. The DUS research is based on international guidelines and is well-harmonised between EU member states. In contrast, the interpretation of the directive for the VCU trials differs between the EU member states because VCU requirements are not defined in detail in the legislation.

VCU trials are obligatory for agricultural crops but not for vegetables, fruit, or ornamental crops. Furthermore, VCU does not apply to a) components of hybrid varieties, b) varieties of grasses not intended for the production of fodder plants, c) varieties for which registration is required solely for export to other than member states. New varieties have to show a clear improvement compared to varieties already on the National List. This improvement can relate to cultivation, usefulness of the crop or usefulness of any product derived from the crop. The selection procedure for new varieties for admission is a stepwise process. The level of admission is mainly determined by the choice of the standard varieties. At the beginning of the testing period, the standard varieties are fixed and do not change during the testing period. About half of the varieties is refused after the first year because of a low agronomic value. Member states can also prohibit the use of a variety if: (1) cultivation of that variety could be harmful to other plant varieties (e.g., too little tolerance for important diseases), or (2) the variety presents a risk to human health (e.g., high mycotoxin level) or the environment, or (3) variety is not suitable because of its type (e.g. if a silage maize variety ripens too late). A breeder can also ask to test a new variety for a specific characteristic or use (e.g. energy maize) with an adapted protocol under specific trial conditions.

Variety trials in Belgium are conducted during at least two different growing seasons at different locations. During a multiple-year testing period, the year effect due to weather conditions is limited and the variation in harvest security parameters (i.e., lodging and stalk rot) between varieties is maximal. The different locations represent the most important soil types for agricultural purposes in Belgium.

1.2.2 Shortcomings of the official variety trials

Variety trials aim to measure the agronomical value of a new variety in a reliable and objective way. The objectivity is partly guaranteed by comparing new varieties with standard reference varieties. Reliability implies that a sufficient number of trials has to be organized during several testing years. Variety trials for silage maize in Belgium are performed in two or three consecutive years on six locations, representing the most important soil types for agricultural purposes. Although an objective conductance by the official institute ILVO is pursued, there are some concerns regarding a proper and scientifically correct assessment of new varieties.

It is clear that only the best varieties are registered on a National List and commercialized. But the introduction of new, potentially very good varieties, is slowed down by the obligatory variety trials. The procedure to register a new variety adds two or three years to the already time-consuming breeding process before a new variety can be introduced on the market. Breeders know the rules for registration before they enter their new varieties in the trials. The criteria for admission are fixed at the beginning of the testing period. Therefore, the evaluation criteria, based on the most important characteristics for agricultural practice, can have a large impact on variety release because they push the breeding companies in a certain direction. However, this also means that innovations from breeding are held back because of the risk that they will never be registered. New ideas require new criteria and new standard references.

1.2.3 Silage maize variety trials in Belgium

The evaluation criteria for silage maize are based on the most important characteristics for agricultural practice. The following characteristics are evaluated during the growing season: early vigour, flowering date, length of the total plant, height and implantation of the ears. Observations for lodging, stalk rot and infection by *Ustilago maydis* are performed just before harvest (no earlier than seven days). Yield and nutritive value are measured at harvest. The main criterion of forage maize nutritive value is OMD predicted by NIRs. Starch concentration is also predicted but more as an indicator of ear proportion and maturation status. A new variety can be registered when the total variety score, based on an index system of all agronomical characteristics, is better than the average of the standard varieties (Van Waes, 2009; Pannecoucque *et al.*, 2015).

The aim of the variety trials is to register varieties relevant for a diversity of local agricultural practices. However, due to practical and financial reasons, variety trials always are imperfect representations of farming practices. An example: varieties differ in maturation rate. In an ideal world each variety should be harvested at its optimal DM concentration, as is recommended to the farming community. However, the procedure for silage maize variety trials in Belgium describes one harvest date for all varieties when the standard reference variety reaches the recommended DM concentration. Upon data processing, varieties are grouped into an early and a late maturity group.

Because nutritive value of silage maize depends on maturity stage, the single harvest date may affect variety ranks. Furthermore, measurements are performed on fresh (i.e. unconserved) maize while mainly maize silage is fed to the animals. The question that arises is "to what respect do varieties with a superior fresh nutritive value maintain their characteristics when ensiled?".

1.3 Incentives, thesis outline, hypotheses and research questions

The incentive of the research reported in this PhD manuscript was a long lasting debate among the maize breeding companies and the conductors of the Belgian National List Variety Trials. Maize breeders and breeding companies obviously are very concerned regarding a proper and scientifically correct assessment of their new varieties.

Points of discussion were: (1) do methods used to measure nutritive value produce relevant and reliable results, (2) does harvest date and plant type interfere with variety ranking of yield and nutritive value of the tested varieties, (3) is there a need to analyse maize silage instead of the current analyses on dried freshly harvested maize.

(1) The main criterion of maize nutritive value in the Belgian National List Variety Trials is OMD. This method is a proxy for what is occurring in the rumen, but e.g. the exposure time to digesting ingredients may differ between the lab and the animal's rumen. Hence it is justified to reassess current methods.

(2) Any harvest date is linked to a particular developmental maturity stage of the silage maize. As maturity rate differs among varieties it is not obvious that a single harvest date values each variety properly. Actual varieties are quite energy dense. While some breeding companies are reaching this target by focusing on a greater ear fraction, other companies focus on a better digestibility of the stover. Modern varieties frequently are stay-green types, i.e. plant types with a delayed maturity of the stover and a potentially longer lasting good digestibility of the stover. Variety trials have been designed in an era when such varieties did not exist. Hence it is justified to question if the current methodology is adapted to a variety of plant types and to study how different plant types can be properly determined.

(3) Current analyses are conducted on dried freshly harvested maize while the animal is eating maize silage. As the composition of the feed is changing by the silage process and may change differentially according to plant type, it seems justified to study how maize nutritive value is changing and how variety ranking is influenced by the silage process.

This PhD research mainly focused on the quality aspects of forage maize and intended to assess and deeply analyse the currently used methods in variety testing and, if necessary, to propose alternative scientifically underpinned new methods to be applied in variety testing.

Hypotheses (H) and research questions (RQ) are listed below and embedded in the general structure of the thesis.

Chapter 1 gives an introduction on forage maize, its relevance for ruminant feeding, the senescence pattern and plant types, the silage process and a description of the official Belgian variety trials.

Chapter 2 summarizes methods and materials used in the following chapters.

Chapter 3 studies how differences in cell wall digestibility (NDFD) and starch influence animal performances and it further investigates which is the best method to estimate NDFD. Results of this study determined which *in vitro* NDFD method was to be used throughout the manuscript.

H1a: Two maize silage varietes (greater NDFD vs more starch) and a third treatment with maize meal to bridge the gap in OMD result in similar performances of dairy cattle

RQ1. "How large is the effect of maize energy source (cell walls versus starch) on milk production?"

RQ2. "How large is the effect of maize energy source (cell walls versus starch) on rumen metabolism, nutrient digestibility and methane emission?"

RQ3. "Can *in vitro* incubation with rumen fluid be used as an alternative for *in situ* nylon bag technique to rank maize silages according to NDFD?"

H1b: Measurements of NDFD are suitable for routine evaluation of the nutritive value

RQ4. "What is the best *in vitro* method to determine NDFD?"

RQ5. "Can NDFD be estimated based on NDF/starch and OMD?"

Chapter 4 focuses on the effect of plant type and maturity on variety performances. Plant types are defined by studying the stay-green trait. This chapter characterizes the selected varieties used in this manuscript.

H2a: Functional SG plant types can be identified by studying photosynthesis and leaf characteristics

RQ6. "Is the variation in photosynthetic capacity (P_{sat}), leaf N concentration, chlorophyll concentration, SPAD and greenness score between varieties large enough to define plant types?"

RQ7. "How large is the effect of the SG trait on photosynthates (sucrose, fructose and starch concentrations) in the leaves?"

RQ8. "How large is the effect of the SG trait on N dynamics and DM yield?"

H2b: Variation in maize nutritive value is mainly determined by maturation and SG trait

RQ9. "How does nutritive value change during maturation?"

RQ10. "How large is the effect of SG trait on maize nutritive value?"

Chapter 5 studies the harvest window searching for the ideal harvest period in order to maximize both yield and nutritive value of forage maize. The concept of a harvest window is introduced.

H3: A single harvest date suffices to compare the nutritive value between varieties when this single harvest date is located within a well-defined harvest window

RQ11. "What is the optimal harvest date, calculated as a compromise between yield, starch concentration and OMD?"

RQ12. "How large is the harvest window, calculated as the set of harvest dates expressing a variety rank that is similar to the variety rank at the optimal harvest date?"

Chapter 6 studies the effect of the silage process on the nutritive value of silage maize. The concept of a harvest window, explained in chapter 5, is used to compare variety ranks of maize silage with variety ranks of fresh material.

H4: A single harvest date without ensiling simulation suffices to compare maize varieties for their nutritive value

RQ13. "How large is the effect of ensiling on maize nutritive value?"

RQ14. "What is the optimal harvest date, calculated from analyses of maize silage?"

RQ15. "How large is the harvest window, calculated as a set of harvest dates expressing a variety rank of fresh maize that is similar to the variety rank at the optimal harvest date calculated from the maize silage?"

Chapter 7 studies the effect of drought on the nutritive value of forage maize.

H5: Drought influences the nutritive value of maize

RQ16. "How large is the effect of drought on maize nutritive value during maturation?"

Chapter 8 brings the general conclusions

2 General materials and methods

BORDER	BORDER	BORDER	BORDER	BORDER	BORDER	BORDER	BORDER	BORDER
KALIENTES	LG30.224	RONALDINIO	BANGUY	NK FALKONE	MAS 17E	LG3220	LG30.222	BORDER
LG30.222	MAS 17E	NK FALKONE	KALIENTES	LG3220	BORDER	BANGUY	LG30.224	RONALDINIO
BORDER	LG3220	BANGUY	RONALDINIO	LG30.224	LG30.222	NK FALKONE	MAS 17E	KALIENTES
BORDER	BORDER	BORDER	BORDER	BORDER	BORDER	BORDER	BORDER	BORDER

2.1 Determination of the nutritive value

This PhD research mainly focused on the nutritive value of forage maize. The energy value of forage maize is widely estimated from the chemical composition and its digestibility. The nutritive value can be measured by standard *in vitro* procedures. However the *in vitro* method to determine cell wall digestibility, expressed as NDF digestibility (NDFD), is more complicated and many versions are described in the literature. A detailed discussion of the choice of *in vitro* NDFD method is given in chapter 3. All parameters used in this manuscript are estimated with near-infrared spectroscopy (NIRs).

2.1.1 Chemical analyses

Chemical composition

Residual moisture was determined by drying at 103 °C. Crude ash was obtained by incineration at 550 °C (ISO, 2002). Crude protein (CP) concentration (Nx6.25) was determined according to Kjeldahl (ISO, 2005). The starch concentration was recorded polarimetrically (ISO, 2000). In determining neutral detergent fibre (NDF), the laboratory procedures given by Goering & Van Soest (1970) were followed, using heat stable amylase and with addition of sodium sulphite and expressed with exclusion of residual ash.

Organic matter digestibility (OMD)

The determination of organic matter digestibility (OMD) was based on *in vitro* digestibility with cellulase (De Boever *et al.*, 1988); a quick and reliable enzymatic method for the prediction of OMD. This method is developed to simulate total *in vivo* digestibility as determined with sheep fed slightly above maintenance energy requirements.

Cell wall digestibility (NDFD)

The standard *in vitro* method determines NDFD by incubating 0.5 g sample with 50 mL buffered rumen fluid at 39 °C for 48 h followed by NDF determination of the undigested residue. The weak points of this method are discussed in chapter 3. This chapter suggests alternative methods or improvements to the standard method. However, the procedure described above continues to be the best practice in determining *in vitro* NDFD. So this standard *in vitro* method is used throughout the manuscript.

2.1.2 Near infrared spectroscopy (NIRs)

NIRs characterizes the entire sample in terms of its absorption properties in the NIR region (De Boever *et al.*, 1997). The NIR technique requires a number of critical steps: acquisition of spectra and reference data, derivation of the regression model and validation of the model. The regression method used to build the calibration equations was the partial least-squares regression. In this regression, the linear combinations used in the prediction equation are obtained by taking both independent (wavelengths) and dependent (composition) variables into account. This method primarily describes the variations of the independent variables which are relevant for modelling the variations of the dependent variables.

A Foss NIRSystems 5000 (Foss, Hillerod, Denmark) and ISIscan (Infrasoft, Port Mathilda, PA, USA) software were used to collect NIRs at 1100 to 2500 nm at 4-nm intervals. Samples were scanned twice, in closed cups. Samples used for the NIR analysis were selected to represent the whole spectral and chemical variability in the target population. The algorithm SELECT was used for efficient selection of the samples, by choosing samples with a minimum standardized Mahalanobis (H) distance of 0.7 from their nearest neighbours.

Statistics relating to NIRs predictions are provided in Table 2.1. Prediction equations for NDF and NDFD of the whole-crop and all quality parameters of the plant parts were developed by the author during this PhD research. Calibration equations for CP concentration, starch concentration and OMD of the whole-crop were provided by the Walloon Agricultural Research Centre in Gembloux (Belgium). Because these calibrations were available, the number of samples is much higher than the number of samples used in the calibrations developed during this PhD.

Table 2.1: Statistics relating to near-infrared spectroscopy (NIRs) predictions of the whole-crop, leaves, stem and ear

Plant part	Parameter	N*	Mean	SEC†	SEV(C)‡	R^2
Whole-crop	Crude protein (g kg^{-1}DM)	6529	76	3.7	3.8	0.899
	Starch (g kg^{-1}DM)	7283	287	16.6	16.8	0.974
	Organic matter digestibility (g kg^{-1}OM)	2902	725	18.9	19.3	0.916
	NDF (g kg^{-1}DM)	192	417	12.8	14.9	0.924
	Cell wall digestibility (g kg^{-1}NDF)	192	633	23.6	28.4	0.816
Leaves	Crude protein (g kg^{-1}DM)	137	81	3.1	3.9	0.979
	Organic matter digestibility (g kg^{-1}OM)	137	560	12.9	16.9	0.923
	NDF (g kg^{-1}DM)	135	687	16.3	19.1	0.952
	Cell wall digestibility (g kg^{-1}NDF)	137	725	20.9	23.4	0.750
Stem	Crude protein (g kg^{-1}DM)	134	25	1.9	2.4	0.943
	Organic matter digestibility (g kg^{-1}OM)	135	426	10.3	13.6	0.978
	NDF (g kg^{-1}DM)	135	667	13.6	17.7	0.972
	Cell wall digestibility (g kg^{-1}NDF)	135	570	22.1	30.7	0.864
Ear	Crude protein (g kg^{-1}DM)	85	82	1.9	2.2	0.977
	Starch (g kg^{-1}DM)	85	569	12.0	15.3	0.973
	Organic matter digestibility(g kg^{-1}OM)	85	904	8.2	10.8	0.893
	NDF (g kg^{-1}DM)	85	287	37.8	49.8	0.810
	Cell wall digestibility (g kg^{-1}NDF)	85	783	38.0	41.9	0.477

* N, number of data points used to develop NIRs calibration

† SEC, standard error of calibration

‡ SEV(C), standard error of cross validation

2.1.3 Statistical analysis

Statistical analyses were performed using the statistical program R (version 3.1.1). Significance was declared at $P < 0.05$. Normality and equal variances were checked with a quantile-quantile (QQ)-plot and Levene's test, respectively.

2.2 Harvest-date trial

2.2.1 Experimental site and design

A harvest-date trial was conducted at three experimental sites (Merelbeke, Bassevelde and Ravels) in Flanders (the northern part of Belgium) during three consecutive years (2013-2015). Different soil types characterized the three sites: sandy loam in Merelbeke, clay in Bassevelde and sand in Ravels. Eight varieties were chosen representing the variation between varieties available on the Belgian market: Banguy (Limagrain), Kalientes (KWS), LG 30.222 (Limagrain), LG 30.224 (Limagrain), LG 3220 (Limagrain), MAS 17E (Maisadour), NK Falkone (Syngenta) and Ronaldinio (KWS).

Their development was monitored using Ontario Units (OU) (Brown, 1969). Daily OU were calculated from minimum and maximum temperatures using linear and quadratic relationships:

$$\mathrm{OU} = \frac{Y_{max} + Y_{min}}{2} \tag{2.1}$$

$$Y_{max} = 3.33(T_{max} - 10) - 0.084(T_{max} - 10)^2 \qquad Y_{max} = 0 \text{ if } T_{max} \leq 10°C \tag{2.2}$$

$$Y_{min} = 1.8(T_{min} - 4.4) \qquad Y_{min} = 0 \text{ if } T_{min} \leq 4.4°C \tag{2.3}$$

Maximum and minimum temperatures necessary to calculate OU were registered in weather stations maximum 20 km way from the experimental fields. The experimental design was a completely randomized block with 3 replicates. Plots consisted of twenty rows with a length of 8 m. Row width was 0.75 m and the plant density was 100 000 plants ha^{-1}. Sowing dates were between 17 April and 7 May (depending on the site and year). Manure, fertilizers and herbicides were applied according to recommended agricultural practices in line with current Belgian regulations.

2.2.2 Weather conditions

The weather conditions at each field trial are presented in Table 2.2. The 2013 growing season was characterized by normal daily average temperatures and normal precipitation in July. In August, rainfall was about 55 mm less than historic normals. In September and October, 45 mm above-average precipitation was measured in Bassevelde and Ravels, while precipitation was normal in Merelbeke. The 2014 growing season was characterized by temperatures that were 2 °C lower than average in August and 2 to 3 °C higher in October. Rainfall was high in July and August: rainfall in July was 20 mm above average and a double amount of rain was measured in August compared with historic normal. In September and October, rainfall was 15 to 45 mm below-average. In 2015, the growing season was characterized by normal temperatures and normal precipitation in July and August but temperatures in September and October were 1 °C lower in Merelbeke and Ravels, and normal in Bassevelde.

Table 2.2: Monthly average temperature and rainfall from July to October in Merelbeke, Bassevelde and Ravels in 2013-2015

		Average temperature (°C)			Historic normals	Rainfall (mm)			Historic normals
		2013	2014	2015	(1981-2010)	2013	2014	2015	(1981-2010)
Merelbeke	July	19.9	19.4	18.6	18.3	87.2	90.5	41.1	70.7
Bassevelde		19.5	19.0	18.8	18.3	72.9	101.4	60.8	80.0
Ravels		20.5	19.3	18.8	18.4	86.6	100.1	39.5	86.4
Merelbeke	August	19.0	16.7	18.8	18.0	16.8	159.7	72.0	72.7
Bassevelde		18.7	15.8	19.9	18.1	27.7	161.3	87.4	82.5
Ravels		19.1	16.6	19.3	17.9	29.6	130.9	118.4	70.4
Merelbeke	September	15.1	16.4	13.6	15.0	70.1	34.4	75.3	69.7
Bassevelde		15.0	15.7	14.9	15.2	122.1	35.5	67.4	80.0
Ravels		15.4	16.2	13.6	14.9	116.3	14.0	85.2	74.7
Merelbeke	October	12.9	14.2	10.4	11.4	83.5	61.4	42.8	77.1
Bassevelde		12.8	13.4	11.8	11.5	144.9	72.9	15.8	93.2
Ravels		12.7	13.8	10.1	10.8	148.8	91.8	41.2	74.7

2.2.3 Harvests and Ontario Units

The harvest dates are defined using OU, cumulative from sowing to harvest, to be able to compare between sites and years. Six harvest dates (H_x) were applied during plant maturation (Table 2.3). At Ravels, a shorter harvest period was applied: harvest dates 1-5 in 2013 and 2015; harvest date 1-4 in 2014. Harvesting was initiated when the kernels of the earliest hybrid, were at the dent stage (R5) (Ritchie *et al.*, 1997) targeting a whole-crop dry matter (DM) concentration of about 25%. The first harvest date in the harvest-date trial coincided with 2546-2664 OU. Subsequent harvests were taken with intervals of one week (64-125 OU), targeting a whole-crop DM concentration of about 40% at the last harvest date.

Table 2.3: Ontario Units (OU) per harvest date, site and year

Harvest	Merelbeke			Bassevelde			Ravels			
date	2013	2014	2015	2013	2014	2015	2013	2014	2015	Mean
H_1	2632	2546	2619	2639	2664	2573	2630	2607	2572	2609
H_2	2749	2698	2735	2750	2800	2691	2747	2737	2697	2734
H_3	2852	2816	2828	2862	2919	2822	2837	2868	2795	2844
H_4	2949	2968	2910	2982	3050	2943	2937	2990	2876	2956
H_5	2987	3085	3005	3023	3149	3054	2968		2938	3026
H_6	3106	3213	3031	3122	3245	3166				3147

At each of the harvest dates, following plants were taken to determine DM yield, DM concentration and nutritive value. A plot of 10 m^2 (approximately 100 plants) was cut by hand 10 cm above soil level and weighted to determine fresh yield. From these 100 plants, five plants were randomly chosen and completely chopped. Another ten representative plants were split into leaves (with husks), stem and ears (dehusked). The ten ears were weighted to determine fresh ear yield. Plant parts were chopped separately. All chopped material (whole-crop and plant parts) was dried at 70 °C for 72 hours and milled over a 1-mm screen using a cutting mill (Retsch Model PK 1000).

3

Maize energy source: starch vs. cell wall

Parts of this chapter are based on: De Boever, J.L., Goossens, K, Peiren, N., Swanckaert, J., Ampe, B., Reheul, D., De Brabander, D.L., De Campeneere, S. and Vandaele, L. The effect of maize silage type on the performances and methane emission of dairy cattle. *Journal of Animal Physiology and Animal Nutrition*, DOI: 10.1111/jpn.12598



3.1 Performances of dairy cattle

3.1.1 Introduction

The greatest part of the feed is fermented in the rumen to volatile fatty acids which deliver most of the metabolisable energy for a lactating cow. Acetic and butyric acid are primarily used as precursors for long-chain fatty acid synthesis, whereas propionic acid mainly as a precursor of glucose (Bannink *et al.*, 2006). According to these authors more starch fermentation will increase propionate production, whereas fermentation of cellulose and hemicellulose stimulate production of acetate and butyrate, respectively. Through its effect on the fatty acid pattern in the rumen, a greater ratio of starch to cell walls in maize silage may reduce methane emission (Hatew *et al.*, 2015). Further, it is very important to optimize the degradation of cell walls in the rumen as their fermentation in the hindgut is limited, and the microbial matter synthesized from neutral detergent fibre (NDF) in the colon will not be absorbed (Van Soest, 1982). Besides the inherent characteristics of the NDF in the feed, the actual degradation of the cell walls depends on the microbial activity in the rumen which is affected by the pH as well as by nitrogen (N)-availability (Dijkstra, 1993). It is assumed that below pH 5.7 NDF is no longer degraded by bacteria and only to some extent by protozoa. In that respect the amount and rate of starch degradation have to be considered.

High starch concentrations in the diet may not only impair degradation of NDF in the rumen, but also digestion of starch in the intestines as found by Van Vuuren *et al.* (2010) with cows fed a ration containing 33% starch on dry matter (DM). Unlike NDF which escapes rumen fermentation, non-structural carbohydrates can be enzymatically digested in the small intestine. Postruminally digested starch can be used more efficiently for milk and body synthesis than starch degraded in the rumen (Nocek & Tamminga, 1991). However, the net glucose absorption by post-stomach tissues is small and the possible significance is mainly through a sparing effect of the utilization of other nutrients like amino acids by the gut (Dijkstra, 1993). The fermentation of carbohydrates in the rumen not only delivers energy to the animal in the form of volatile fatty acids, but also supplies ATP for microbial protein production. The efficiency of microbial synthesis appears clearly greater for degraded starch than for degraded NDF, with respectively 277 and 172 g microbial protein per kg substrate (Van Duinkerken *et al.*, 2011).

The objective of the experiment with dairy cows was to test the hypothesis **H1a: two maize silage varieties (greater NDFD vs more starch) and a third treatment with maize meal to bridge the gap in OMD result in similar performances of dairy cattle** by answering three research questions: (**RQ1**) "How large is the effect of maize energy source on milk production?"; (**RQ2**) "How large is the effect of maize energy source on rumen metabolism, nutrient digestibility and methane emission?"; (**RQ3**) "Can *in vitro* incubation with rumen fluid be used as an alternative for *in situ* nylon bag technique to rank maize silages according to NDF digestibility (NDFD)?" (Figure 3.1).

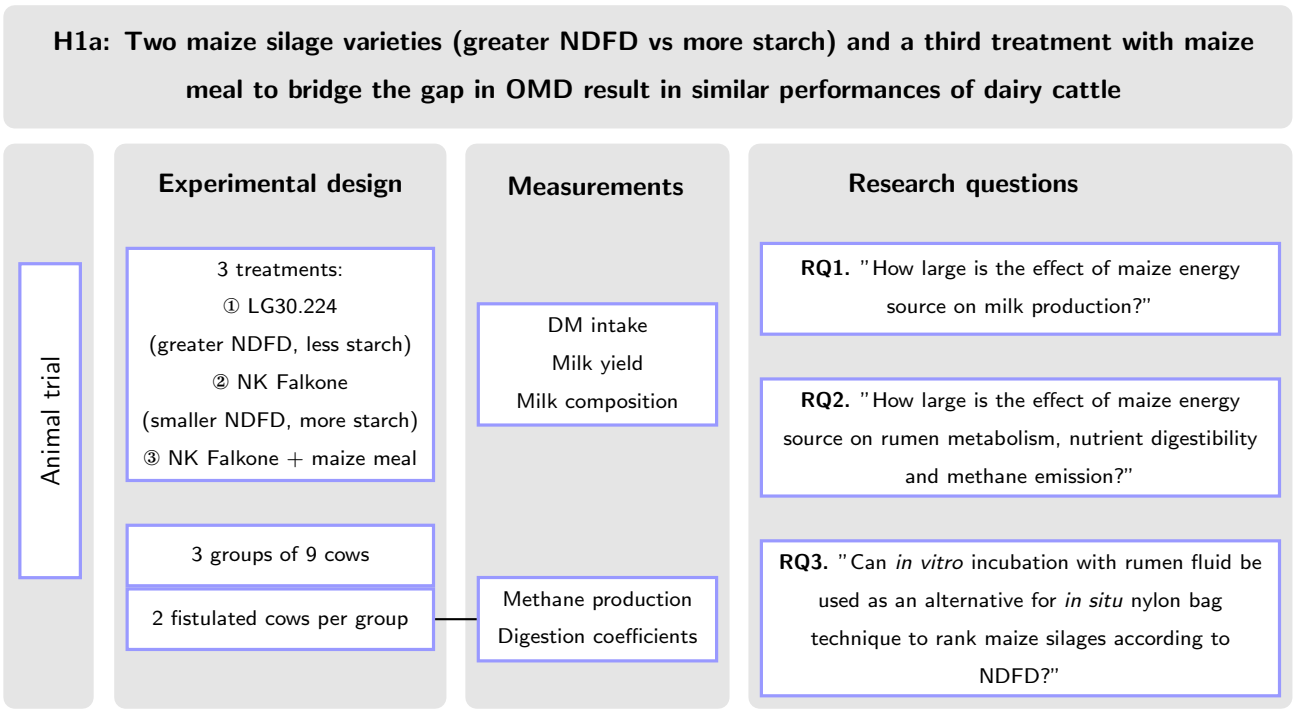


Figure 3.1: Schematic presentation of the research linked to H1a. All measurements were applied to all experimental material, except for the relation indicated by a line. When a line is shown, performances were limited to the relation indicated by the line.

3.1.2 Materials and Method

Maize silage types and treatments

To obtain the two types of maize silage we selected two varieties of whole crop maize per type based on the starch concentration and organic matter digestibility (OMD) as presented by the Belgian Descriptive and Recommended Variety List (2012) as well as based on our own results of *in vitro* NDFD. The two varieties with high starch concentration and low NDFD were NK Falkone and LG30222, whereas the two varieties with a smaller starch concentration and high NDFD were Ronaldinio and LG30224. The 4 maize varieties were sown on 27 April 2013 on a homogenous field of about 8 ha. On 4 October 2013 about 1 ha of each of the 4 varieties was harvested at about 35% DM with a maize chopper set at a theoretical length of 7 mm provided with rolls and ensiled in a clamp silo. Based on the analyses of silage samples taken at two and four months after ensiling, we decided to use NK Falkone as variety with low NDFD and high starch and LG30224 as variety with high NDFD and low starch. NK Falkone and LG30224 clearly differed in NDFD: 51.6 and 60.9% respectively, whereas their difference in starch concentration was rather limited: 381 versus 355 g kg^{-1}DM, respectively. LG30224 also showed a greater *in vitro* OMD by about 2% units, resulting in a greater predicted concentration of net energy lactation (NE_L). The silages of NK Falkone and LG30224 are further referred to as treatments FA and LG respectively. To enable the comparison of both silages on a similar energy basis, a third treatment was introduced by supplementing FA with maize meal (FA+MM) in a ratio of 92/8 on DM to bridge the gap in OMD and NE_L and to increase the difference in starch concentration.

Animals and feeding

The dairy cow experiment was carried out in the period from February to May 2014 in accordance with the Belgian law for care of experimental animals (Royal Decision 14.05.2010) and approved by the Animal Ethics Committee of ILVO (Dossier 2014/215). The feeding trial was set up according to a Latin square design with three groups of nine Holstein-Friesian cows and three periods of three weeks, with the first week for adaptation and the last two weeks as experimental period. The maize silage in the three treatments was combined with wilted grass silage in a ratio of 65/35 on DM to limit starch concentration to maximum 25% in the ration DM. Roughage was fed ad libitum and was supplemented with concentrates. During a pre-experimental period of two weeks all animals received a same MS (different from the experimental silages) and their roughage intake, milk production and composition were registered. Then, three similar groups were made based on lactation number, milk production, fat and protein concentration of milk, total dry matter intake (DMI), body weight and days in milk. At the beginning of the trial these parameters were on average for all cows: 2.3 lactations, 33.1 kg milk, 4.14% fat, 3.38% protein, 20.87 kg DMI, 638 kg body weight and 118 days in milk. One animal had to be removed from the trial because of gastro-intestinal problems. Within each group a cow with a rumen cannula (internal diameter of 10 cm; Bar Diamond Inc, Parma ID, USA) was assigned. The supply of concentrates at the start of the trial was calculated individually based on the ad libitum roughage intake during the pre-experimental period and according to 100% of the animal requirements for NE_L (Van Es, 1978), to 105% of the requirements for true protein digested in the intestines (DVE) and to a degraded protein balance (OEB) of 175-200 g day^{-1} (Van Duinkerken *et al.*, 2011). The combination of maize silage and grass silage ensured that minimum physical structure requirements were covered (De Brabander *et al.*, 1999). The concentrates concerned a compound feed with a normal crude protein (CP) concentration, another with low CP, untreated and formaldehyde treated soybean meal as well as urea. All feeds were fed in two meals at 8.30 and 17.30 h, starting with maize silage (including maize meal for the third treatment), followed by urea, grass silage and finally the concentrates. All animals were housed individually in a tie-stall with rubber bedding, separate mangers and continuous access to water.

Measurements and sampling

All feeds were weighed individually per meal. DMI was evaluated daily to ensure ad libitum intake and to prevent large amounts of residues. Leftovers were collected and weighed at least once per week and, if more than 2 kg, were analysed for DM concentration. Cows were weighed on two consecutive days at the end of the three trial periods. Animals were milked twice daily at 5.30 and 17.30 h. Milk yield was recorded at each milking and milk from the last four milkings of the last week from the pre-experimental period and of the two last weeks from each trial period was sampled for analysis for fat, protein, lactose and urea with FTIR (Lactoscope Advanced, Delta Instruments, Drachten, The Netherlands). Milk production was corrected for fat and protein with the formula:

$$\text{fat-protein corrected milk} = [0.337 + (0.116 \times \%\text{fat}) + (0.06 \times \%\text{protein})] \times \text{milk production} \quad (3.1)$$

During the last two weeks of the three trial periods all feeds were sampled once per week. Samples from the maize silages and the grass silage were pooled per period, whereas samples from the other feeds were pooled for the whole trial. During the first two days of the last week of each trial period, samples of rumen fluid were taken from the three fistulated cows at 8.00 h (just before the morning meal) and at 1, 2, 4, 6.5 and 9 h thereafter. Further during the last four days of each trial period, two cows from each group with among them the fistulated cow, were put in open-circuit gas exchange chambers to measure methane production (De Campeneere & Peiren, 2012). Before entering the chambers the cows received a urine catheter to separate urine and faeces, which were totally collected. Each morning individual faeces were weighed, homogenized and a 1% sample put in the freezer at -18 °C. Pooled samples were analysed to determine the digestion coefficients of organic matter (OM), CP, crude fibre, crude fat, starch and NDF.

In situ rumen degradation characteristics and protein value of feeds

The rumen degradability characteristics of OM, CP, starch and NDF were determined with the nylon bag technique (CVB, 2004) using three rumen cannulated cows. The cows (different from those involved in the feeding trial) produced at least 15 kg of milk and were fed a basal ration consisting of grass silage and maize silage (50/50 on DM-basis) in two meals supplemented with concentrates to meet their requirements for energy and protein. Nylon bags (Sefar, Heiden, Switzerland) measuring 8x10 cm and with a pore size of 37 μm were filled with 2.5 or 5 g DM-equivalent of the feed and were then heat-sealed. The roughages were frozen and finely cut (particles $\leq$ 1 cm), whereas the concentrates were ground through a 3 mm screen. The bags were incubated in the rumen during 8, 24, 48, 72 and 336 h for the three roughages and during 3, 8, 24, 48 and 336 h for the two concentrates. For 3 and 8 h, two bags per cow (6 bags per feed) and for the other incubation times three bags were incubated; for 72 and 336 h with a double sample weight. Besides, 3 bags, filled with sample, were not incubated in the rumen, but underwent all other treatments to determine the washout fraction (W). Bags were incubated just before the morning meal. After incubation, bags with residues were immediately immersed in ice water, further rinsed under running tap water and put in the freezer (-18 °C). After collection of all bags, they were machine-washed (Zanussi, Frankfurt/Main, Germany) with cold water without spin cycle and then freeze-dried. Residues from the three cows were pooled per incubation time and ground to pass a 1-mm screen (Retsch ZM-1) for analysis of moisture, crude ash, CP and NDF. The potentially degradable fraction (D) was calculated as 100 - W - U, with U being the undegradable fraction after 336 h of incubation. The degradation rate of D (kd_D) was derived by iteration using the exponential model $d(t) = W + D \times (1 - e(-kd_D \times t))$ with d(t) the disappearance at time t (Ørskov & McDonald, 1979). The obtained degradation characteristics for starch and NDF of the maize silages were used to calculate the effective rumen fermentability of starch (%FSTA) and NDF (%FNDF) using the formulae:

$$\%FSTA = W \times [kd_W/(kd_W + 8)] + D \times [kd_D/(kd_D + 6)] \tag{3.2}$$

with $kd_W = 2\ kd_D + 37.5$ and 8 and 6 the passage rate (kp) of W and D, respectively.

$$\%FNDF = D \times [kdD/(kd + kp)] \tag{3.3}$$

with $kp = 1.39 + 0.1775 \times kd$

Further, the degradation characteristics of the nutrients were used to calculate the rumen fermentability of OM, the DVE and OEB concentration of the roughages according to the Dutch protein evaluation system (Van Duinkerken *et al.*, 2011). The protein value of the concentrates were estimated based on chemical composition and tabular values (CVB, 2011).

Analyses

Samples of feeds and faeces were dried in a ventilated oven at 65 °C and then ground through a 1-mm screen (Wiley, Rheotec, Maarkedal, Belgium). Residual moisture was determined by drying at 103 °C. Crude ash was obtained by incineration at 550 °C. Crude protein (Nx6.25) was determined according to Kjeldahl. Crude fat was extracted with petroleum-ether after hydrolysis with hydrochloric acid Crude fibre was obtained with the Ankom Fiber Analyser (Ankom Technology, Macedon NY, USA) after boiling subsequently with sulfuric acid and sodium hydroxide. NDF was determined with the Ankom Fiber Analyser using α-amylase and sodium sulfite and expressed on ash-free basis (Van Soest *et al.*, 1991). Starch was determined after autoclaving and hydrolysis with amyloglucosidase. Sugars were extracted with 40% ethanol and analysed according to the Luff Schoorl method. The silage fermentation products of the three silages were determined on a water extract; lactic acid by an enzymatic method (Bergmeyer & Gawehn, 1974; Noll, 1966), acetic acid, propionic acid, butyric acid and alcohols with gas chromatography (Jouany, 1981) determined. The DM concentration and the chemical composition of the silages were corrected for losses of volatile substances during drying (Dulphy & Demarquilly, 1981). The OMD was determined *in vitro* using cellulase (De Boever *et al.*, 1988). *In vivo* OMD and NE_L of the feeds were predicted with regression equations based on *in vitro* OMD and chemical composition (De Boever *et al.*, 1999). The NDFD was determined by in vitro incubation of 0.5 g feed sample with buffered rumen fluid during 48 h at 39 °C, analogue to the first step of the method from Tilley & Terry (1963), followed by NDF-analysis of the residue. The structure value of the roughages was derived from crude fibre and the structure value of concentrates was derived from crude fibre and rumen undegraded starch (De Brabander *et al.*, 1999). Rumen fluid samples were filtered through sieve gauze and pH was measured immediately. Volatile fatty acids were analysed by gas chromatography (Getachew *et al.*, 2002), whereas ammonia was analysed by the micro-diffusion method of Voight & Steger (1967).

Statistics

Data were analysed in Statistica 12 (2015) using a mixed effects ANOVA with treatment and period as fixed factors and cow as random factor. In case of a significant treatment effect ($p < 0.05$), means were compared using a Tukey post-hoc significant difference test ($p < 0.05$).

3.1.3 Results

Chemical composition and nutritive value of the feeds

The chemical composition of the two maize silages used in the feeding trial is presented in Table 3.1. The values represent the mean and SD from three pooled samples taken during each of the three trial periods. The DM concentration of FA was 4.5% greater than that of LG. The difference in starch concentration between the two silages was 19 g kg^{-1}DM. The LG showed greater NDFD and OMD, by 4% units and 1.5% units, respectively, resulting in 0.16 MJ more NE_L per kg DM. The DVE and structure value of both silages were similar, whereas the OEB was less negative for FA.

Table 3.1: Nutritive value of maize silages in the feeding trial

	LG*	FA†
Dry matter (%)	35.9 ± 0.5	40.4 ± 0.4
Crude protein (g kg^{-1}DM)	70 ± 0.4	74 ± 1
Starch (g kg^{-1}DM)	363 ± 9	382 ± 31
NDF (g kg^{-1}DM)	372 ± 17	395 ± 17
ADF (g kg^{-1}DM)	205 ± 3	217 ± 3
ADL (g kg^{-1}DM)	20 ± 1	26 ± 4
OM digestibility (g kg^{-1}OM)	750 ± 3	735 ± 3
Cell wall digestibility (g kg^{-1}NDF)	551 ± 27	511 ± 25
NE_L (MJ kg^{-1}DM)	6.46 ± 0.03	6.30 ± 0.04
DVE (g kg^{-1}DM)	59	58
OEB (g kg^{-1}DM)	-50	-45
Structure value (kg^{-1}DM)	1.50	1.55

* LG, maize silage LG30.224; † FA, maize silage NK Falkone

The rumen degradation characteristics (Table 3.2) show that OM, protein and NDF of FA have a greater undegradable fraction and that all nutrients are slower degraded in comparison with LG. This results in a smaller effective fermentability of OM, protein, starch and NDF for FA by 6.8, 8.2, 8.4 and 4.3% units, respectively.

Table 3.2: Rumen degradation characteristics for the two maize silages

	Organic matter		Protein		Starch		NDF	
	LG*	FA†	LG*	FA†	LG*	FA†	LG*	FA†
Washable fraction (%)	35.7	34.0	51.3	61.3	53.0	60.2	0.0	0.0
Degradable fraction (%)	48.3	47.1	31.5	15.6	47.0	39.9	68.4	64.8
Undegradable fraction (%)	16.1	19.0	17.2	23.1	0.0	0.0	31.6	35.3
Degradation rate (% h^{-1})	6.52	4.55	10.7	3.78	16.3	6.97	3.69	3.06
Effective fermentability (%)	59.7	52.9	50.8	42.6	81.9	73.5	44.0	39.7

* LG, maize silage LG30.224; † FA, maize silage NK Falkone

Nutrient intake and effects on dry matter intake and milk production performances

Table 3.3 shows the chemical composition and nutritive value of the ration in the three treatments as calculated from the effective intake and the characteristics of the individual feeds. The average body weight of the animals at the end of the experiment was similar for the three groups and amounted to 640, 637 and 640 kg, respectively. The three rations showed relative small differences in chemical composition. Starch concentration was small for LG and great for FA+MM, whereas NDF concentration was greater for FA than for both other rations. The ration with FA contained less NE_L than the rations with LG and FA+MM. In general, the provision with NE_L was just above the animal requirements. The DVE concentration of the three rations was similar, but the supply was clearly (more than 20%) above the requirements. The degraded protein balance was below the formulated target of 175 g d^{-1}, with the greatest OEB for FA and the smallest for LG. The structure value of the ration was on average 1.7 kg^{-1}DM, well above the minimum animal requirements of about 1.0 kg^{-1}DM.

Table 3.3: Chemical composition and nutritive value of the rations based on the effective intake of the feeds (means ± SD of 26 cows)

	LG*	FA†	FA+MM‡
Crude protein (g kg^{-1}DM)	161 ± 11	164 ± 11	162 ± 11
Crude fat (g kg^{-1}DM)	19 ± 0.9	21 ± 0.6	22 ± 0.5
Starch (g kg^{-1}DM)	204 ± 10	211 ± 14	226 ± 14
Sugars (g kg^{-1}DM)	49 ± 11	50 ± 10	49 ± 10
NDF (g kg^{-1}DM)	358 ± 25	369 ± 25	359 ± 25
NE_L (MJ kg^{-1}DM)	6.61 ± 0.17	6.54 ± 0.17	6.62 ± 0.16
NE_L (supply requirements^{-1})	1.01 ± 0.08	1.00 ± 0.07	1.03 ± 0.09
DVE (g kg^{-1}DM)	100 ± 12	100 ± 13	101 ± 13
DVE (supply requirements^{-1})	1.23 ± 0.13	1.23 ± 0.09	1.26 ± 0.09
OEB (g d^{-1})	90 ± 41	146 ± 32	113 ± 40
Structure value (kg^{-1}DM)	1.68 ± 0.21	1.71 ± 0.20	1.66 ± 0.19

* LG, maize silage LG30.224; † FA, maize silage NK Falkone

‡ FA+MM, maize silage NK Falkone + maize meal

The effect of treatment on DMI and production performances is presented in Table 3.4. Daily DMI of the maize silage did not differ between LG and FA but was significantly greater than for FA+MM, which included on average 0.79 kg DM maize meal. The DMI of all roughages and of all feeds was significantly greater for treatment FA+MM than for FA with for LG a value in between. Maize silage made up about 47% of total DMI. Milk production was not significantly affected by treatment, while fat-protein corrected milk was significantly greater for LG than for FA. Treatment had no effect on milk composition nor on the daily production of fat, protein or lactose. Milk urea concentration was significantly greater for both rations with FA than with LG, the difference amounting to about 30 mg dL^{-1}. N-efficiency and weight change also showed no differences among treatments.

Table 3.4: Dry matter intake and production performances (means of 26 cows during 3 periods of 2 weeks)

	LG*	FA†	FA+MM‡	S.E.	*P* value
Dry matter intake (kg d^{-1})					
Maize silage	9.69^{a}	9.49^{a}	9.16^{b}	0.122	< 0.001
Grass silage	5.07	5.04	5.16	0.069	0.087
Roughages total	14.77^{a}	14.53^{ab}	14.32^{b}	0.189	0.009
Maize meal	-	-	0.79		< 0.001
Concentrates	5.80	5.78	5.69	0.181	0.091
Total ration	20.56^{ab}	20.32^{b}	20.81^{a}	0.257	0.009
Production performances					
Milk (kg d^{-1})	29.5	29.0	29.4	0.61	0.074
Fat-protein corrected milk (kg d^{-1})	30.5^{a}	29.9^{b}	30.3^{ab}	0.59	0.018
Fat (%)	4.33	4.30	4.32	0.034	0.70
Protein (%)	3.37	3.33	3.38	0.023	0.11
Lactose (%)	4.71	4.71	4.73	0.012	0.21
Urea (mg dL^{-1})	235^{b}	265^{a}	261^{a}	3.6	< 0.001
Fat (g d^{-1})	1265	1237	1253	24.8	0.055
Protein (g d^{-1})	981	956	962	18.3	0.15
Lactose (g d^{-1})	1423	1406	1423	29.1	0.25
N-efficiency ($100 \times N_{milk}\ N_{intake}^{-1}$)	29.3	28.9	29.0	0.43	0.46
Weight change (kg d^{-1})	-0.16	0.04	-0.08	0.054	0.26

means in a row with the same superscript letter are not significantly different

* LG, maize silage LG30.224; † FA, maize silage NK Falkone; ‡ FA+MM, maize silage NK Falkone + maize meal

Rumen fermentation, nutrient digestibility and methane production

The effect of maize silage type on rumen fermentation was examined with three rumen cannulated cows. One cow was 87 days in milk at the start of the experiment, whereby daily fat-protein corrected milk decreased from 30.1 kg during the last week in period 1 to 27.5 kg in the last week of period 3. The other cows were both 221 days in milk and end lactation with a decrease of fat-protein corrected milk during the experiment from 18.1 to 9.9 kg d^{-1} and from 14.4 to 8.7 kg, respectively. The pH, ammonia and molar proportions of acetic, propionic and butyric acid in the rumen fluid of the 3 cows sampled just before the morning meal and at different time intervals thereafter are given in Table 3.5. There was a cow effect for pH at all sampling times, with the high producing cow showing significantly smaller values than the other cows, on average 6.34 vs. 6.84 and 6.80. The same cow also showed greater concentration of ammonia at 0 h (11.8 vs 8.3 and 7.9 mL 100 mL^{-1} rumen fluid). Molar ratios of volatile fatty acids were not affected by cow, except for the proportion of butyric acid which was significantly smaller 9 h after the morning meal for one of the low producing cows, which could be considered without much relevance. Treatment had no significant effect ($p > 0.05$) on any of the measured rumen fermentation parameters.

The pH was highest just before the morning meal (overall mean: 6.98) and decreased gradually to a minimum 6.5 h after feeding (overall mean: 6.54) and then raised again before the evening meal. The ammonia concentration was low just before the morning meal (overall mean: 9.3 mL 100 mL^{-1}) and increased very fast to a peak level 2 h after feeding (overall mean: 24.3 mL 100 mL^{-1}) and then decreased very fast to a minimum concentration 6.5 h after feeding (overall mean: 7.9 mL 100 mL^{-1}). The proportion of acetic acid in total VFA was three times greater than that of propionic and butyric acid, with for both very similar percentages.

Table 3.5: pH, ammonia and volatile fatty acid concentration in rumen fluid before the morning meal (0 h) and at 5 times thereafter (means of 3 cows x 2 d)

		LG*	FA†	FA+MM‡	S.E.	*P*-T#	*P*-C#
pH	0 h	6.96	6.94	7.05	0.05	0.80	0.031
	1 h	6.73	6.67	6.61	0.06	0.87	0.005
	2 h	6.57	6.58	6.60	0.08	0.99	< 0.001
	4 h	6.52	6.56	6.64	0.07	0.88	0.028
	6.5 h	6.55	6.56	6.51	0.07	0.97	0.023
	9 h	6.59	6.55	6.67	0.08	0.92	0.035
	mean	6.65	6.64	6.68	0.06	0.60	< 0.001
Ammonia	0 h	8.35	10.36	9.23	0.55	0.60	0.018
(mg 100mL^{-1})	1 h	22.41	23.31	23.53	1.18	0.96	0.070
	2 h	24.14	24.24	24.47	0.73	0.99	0.50
	4 h	11.77	20.19	16.08	1.56	0.35	0.16
	6.5 h	7.43	7.70	8.60	0.92	0.93	0.56
	9 h	7.81	9.20	8.42	0.75	0.80	0.13
	mean	13.65	15.83	15.05	0.44	0.57	0.83
Volatile fatty acids (mol%)							
Acetic acid		59.03	60.71	59.72	0.35	0.44	0.72
Propionic acid		18.90	18.33	18.75	0.28	0.82	0.61
Butyric acid		17.96	17.68	18.26	0.22	0.67	0.49

* LG, maize silage LG30.224; † FA, maize silage NK Falkone; ‡ FA+MM, maize silage NK Falkone + maize meal; # *P* values of treatment (T) and cow (C) effect

Table 3.6 presents the digestion coefficients of the nutrients as well as the methane emission obtained with the 3x2 cows during their stay in the gas exchange chambers. The six cows in the chamber study produced on average 6.4 kg less fat-protein corrected milk than the 26 cows in the feeding trial. Their total daily DMI was on average 2.2 kg smaller, mainly due to the provision of less concentrates. The relative proportions in intake and fat-protein corrected milk production among treatments remained similar to those from the feeding trial. Treatment had no significant effect ($P > 0.05$) on the digestion coefficients of the total ration with exception of crude fat which was better digested with FA than with LG. Methane emission per day and expressed per kg NDF was greater with LG than with FA, but significant differences disappeared when methane emission was expressed per kg DMI or per kg fat-protein corrected milk.

Table 3.6: Nutrient digestibility and methane emission (mean of 6 cows during 3 periods of 1 week) of the whole ration

	LG*	FA†	FA+MM‡	S.E.	*P* value
Dry matter intake (kg d^{-1})	18.7	17.7	18.9	0.6	0.12
Fat-protein corrected milk (kg d^{-1})	24.1	23.5	24.0	2.1	0.85
Weight change (kg d^{-1})	-0.66	0.10	-0.12	0.17	0.28
Digestibility (g kg^{-1})					
Organic matter	741	733	744	6	0.38
Crude protein	690	689	686	8	0.93
Crude fat	624^{b}	674^{a}	658^{ab}	12	0.023
Crude fibre	629	622	629	8	0.80
NDF	598	599	609	8	0.80
Starch	977	973	976	2	0.61
Methane emission					
CH_4 (g d^{-1})	376^{a}	336^{b}	359^{a}	14	0.011
CH_4 (g kg^{-1}DM)	20.2	19.0	19.0	0.4	0.09
CH_4 (g kg^{-1}NDF)	52.9^{a}	48.8^{b}	50.3^{b}	1.7	0.016
CH_4 (g kg^{-1}Fat-protein corrected milk)	18.5	15.5	17.5	1.7	0.50

means in a row with the same superscript letter are not significantly different

* LG, maize silage LG30.224; † FA, maize silage NK Falkone; ‡ FA+MM, maize silage NK Falkone + maize meal

3.1.4 Discussion

Chemical composition and nutritive value of the feeds

In our comparison of two different types of maize silage, we achieved the aimed difference in cell wall digestibility (4% units), whereas the aimed difference in starch concentration was only partly reached, as a difference of 19 g kg^{-1}DM is relatively small. Although both genotypes were sown, grown and harvested on the same parcel and in the same conditions, FA contained 45 g kg^{-1} more DM than LG. In fact, the difference in maturity pronounced the genetic differences. The greater DM concentration of FA can explain the smaller concentration of fermentation products and in its turn the greater concentration of the other nutrients, including starch. The later developmental stage of variety NK Falkone may explain the slower rumen degradation of its nutrients. Concerning the degradation of starch and protein, it was shown that in more mature maize the vitreousness of the endosperm increases, whereby zein proteins cross-link and encapsulate starch into a hydrophobic starch-protein matrix (Hoffman *et al.*, 2011). The more advanced development at harvest of NK Falkone may also partly explain the smaller NDF fermentability of FA because of a greater degree of lignification (Barrière *et al.*, 2004). Indeed, ADL concentrations were 30% greater for NK Falkone compared to LG30.224 (26 vs 20 g kg^{-1}DM).

In situ rumen NDF-fermentability was more than 10%-units smaller than *in vitro* NDFD, but the difference between LG and FA was similar with both methods, respectively 4.0% and 4.3%. Moreover, the degradation of NDF after 48 h rumen incubation amounted to 53.7 and 49.7% for LG and FA, respectively, thus very similar to the 48 h *in vitro* NDFD. This means that *in vitro* incubation with rumen fluid can be used as a more convenient, rapid and cheaper alternative for in the *in situ* nylon bag technique to rank maize silages according to NDFD. Oba & Allen (1999) stated that NDFD *in vitro* or *in situ* is a better measure of forage quality than *in vivo* NDFD, because the latter is confounded by different retention times and digestibility in the large intestine may reduce differences.

Dry matter intake and performances

The greater DMI of the ration with FA+MM can be explained by the smaller substitution rate of the meal than of the silage, which replaced part of FA. The type of maize silage did not significantly affect the intake of maize silage nor total DMI. One could expect a greater intake from a maize silage with a greater DM and starch concentration, which is usually associated with a smaller NDF concentration (Khan *et al.*, 2014). However in our experiment, FA contained not only more starch but also more NDF than LG. Further, from a review of 10 studies (Khan *et al.*, 2014) appeared that DMI of maize silage increases with DM concentration up to 350 g kg^{-1}, but then declines slightly beyond that DM concentration. Moreover, compared with LG, the concentration of NDF in FA was not only greater, but its digestibility was significantly smaller. Oba & Allen (1999) statistically evaluated 13 sets of forage comparisons with dairy cows and found per percentage greater *in vitro* or *in situ* NDFD an increase of 0.17 kg in DMI and of 0.25 kg in 4% fat corrected milk. In our experiment, the total DMI with LG was only 0.24 kg and not significantly greater than with FA, thus far below the increase in DMI of 0.68 kg which could be expected from the study of Oba & Allen (1999). On the other hand, cows fed with LG produced 0.6 kg significantly more fat-protein corrected milk than with FA or an increase of 0.15 kg milk per %-point greater NDFD, which is also smaller than the increase found by Oba & Allen (1999). The greater milk production with LG can not only be explained by the greater intake, but also by the greater NE_L provision as compared with FA (difference of 0.16 MJ kg^{-1}DM). This is supported by the third treatment whereby addition of maize meal to FA bridged the energy gap between the two maize silages and resulted in similar standard milk production and milk composition.

Because the review of Oba & Allen (1999) concerned different types of forages, we looked further for comparative dairy cow trials with maize silage as main forage in the ration. Weiss & Wyatt (2002) compared silages of two maize hybrids at 45% in the ration DM, the first with a 4.7% units greater 30-h *in vitro* NDFD and a 44 g kg^{-1}DM smaller starch concentration than the second and found no differences in total DMI (23.7 kg d^{-1}), 4% fat corrected milk (33.3 kg) or milk composition. The lack of effect was ascribed to the appreciably greater NDF of the first silage (490 vs. 424 g kg^{-1}DM). Ivan *et al.* (2005) compared two maize silages, the first with a 4.1% units greater 30-h *in vitro* NDFD and 32 g kg^{-1}DM smaller starch concentration than the second at equal NDF (308 g kg^{-1}DM) and similar starch concentration (321 vs. 311 g kg^{-1}DM) in the ration.

They observed no significantly greater (P=0.32) DMI (27.1 vs. 26.5 g d^{-1}), but a significantly greater 4% fat corrected milk production (34.9 vs. 33.4 kg; P=0.03) and milk fat concentration (3.91 vs. 3.79%, P=0.07). This effect was ascribed to the better total tract NDFD (46.9 vs. 36.5%) and a trend for increased rumen turnover of NDF as measured by rumen evacuation (6.55 vs. 5.09% h^{-1}). The greater milk urea concentration with FA than with LG is due to the greater OEB concentration of the former, which in turn could be explained by the greater CP concentration and the greater washable CP fraction. The N-efficiency (N_{milk} N^{-1}_{intake}), which was not affected by treatment, reached almost 30%. This is relatively high considering the more than 20% extra supply of DVE above the animal requirements, but may be explained by the low OEB level as well as by the high milk production of the animals.

Rumen fermentation, nutrient digestibility and methane emission

Because the effect of maize silage type on rumen fermentation was studied with only one cow per group and the effect on nutrient digestibility and methane emission with two cows per group, these results should be considered rather indicative than conclusive. The type of maize silage did not affect pH nor volatile fatty acid composition. The minimum pH measured during our experiment was 6.0 for the high producing cow fed FA. This is far above the threshold value of 5.7, below which negative effects on NDFD may be expected (Dijkstra, 1993). The lower pH of this cow as compared with the other two cows is due to the smaller roughage proportion in the ration amounting to 75 and 93%, respectively and proves that the experimental rations were formulated with a safe roughage proportion. In accordance, Weiss & Wyatt (2002) and Ivan *et al.* (2005) neither observed an effect on pH or volatile fatty acid composition. In the latter study the mean daily pH was much lower (5.95) than in our study (6.65), which could be related to the difference in forage proportion in the ration DM, being 55 and 70%, respectively and to smaller forage proportion in the ration DM (55%) and to the greater production level of the cows. Treatment had also no effect on total tract digestibility of the nutrients, with exception of crude fat. The better fat digestibility of the ration with FA may be explained by its greater fat concentration. The greater rumen NDFD of LG as compared with FA did not result in any difference in total tract NDFD of the ration. Weiss & Wyatt (2002) even found a smaller total tract NDFD of the ration with the maize hybrid fed at 45% of the ration DM, showing greater 30-h *in vitro* NDFD. This discrepancy was explained partly by dilution (maize silage made up less than 50% of the ration DM) and partly by the faster rumen passage rate of the maize silage with better rumen NDFD. The absence of a treatment effect on total tract digestibility of starch, which showed a high overall mean value of 97.5%, proves that the greater amount of bypass starch with FA had no negative effect on postruminal digestion.

The ration with LG resulted in greater methane production per day than that with FA. The proportions between treatments remained the same when expressed per kg DMI or per kg fat-protein corrected milk, but the differences were no longer significant because of the greater DMI and fat-protein corrected milk with LG. Further, CH_4 kg^{-1}fat-protein corrected milk showed greater individual variation due the low milk productions of the two fistulated cows. The smaller methane production with FA than with LG can be explained partly by the smaller rumen fermentability of NDF (Table 3.2: 39.7 vs. 44.0%) and partly by its greater DM concentration resulting in more starch bypassing rumen fermentation. This finding is in agreement with Hatew *et al.* (2015), who found reduced CH_4 emission per kg DMI and per kg fat-protein corrected milk with increasing maturity of maize silage caused by a greater starch and smaller NDF concentration as well as by a smaller rumen degradability of both starch and NDF.

3.1.5 Conclusion

In conclusion, our feeding experiment showed that type of maize silage did not affect milk production performances, on the condition that the difference in OMD can be compensated by increasing diet starch concentration, without compromising the provision of sufficient physical structure to avoid negative effects on rumen fermentation. Our study also supports the findings that maize silage containing more bypass starch produces less methane. Our results further support the view that one can breed maize varieties of high nutritive value by different ways, either by a greater starch concentration or by improving NDFD. In maize variety lists both OMD and starch concentration are important criteria to take account in ration formulation.

3.2 Cell wall digestibility

3.2.1 Introduction

Forage maize digestibility is greatly affected by the concentration of NDF and NDF digestibility (NDFD). For practical and financial reasons, digestibility assessments are performed using *in vitro* tests. But this approach is only valid if the *in vitro* method is a good predictor of *in vivo* digestibility. Oba & Allen (1999) stated that NDFD measured *in vitro* is a better indicator for the potential energy value of forages than NDFD measured *in vivo* because *in vivo* measurements can be confounded by different retention times in the rumen. The *in vitro* measurement of NDFD is accomplished by a multiple-step procedure including drying and grinding of the sample; subsampling for dry matter (DM), NDF and NDFD analyses; fermentation of a subsample; and NDF analysis of the fermentation residue (Hall & Mertens, 2012). Each step contributes to the normal variation in the analytical values.

Repeatability and reproducibility of the *in vitro* NDFD method are affected by several factors. The fermentation depends on the incubation time. The initial degradation phase is the most sensitive to different conditions because cellulolytic microbes have to adhere to the fibrous material, delaying the fibre degradation process and extending the incubation time from 30 h to 48 h allows for a more complete NDF degradation (Spanghero *et al.*, 2010). Changing the expression of NDF degradation as a percentage of NDF (NDFD) to a percentage of DM (dNDF) decreases the variation in the analytical values. The assay error made in determining NDF is included twice in the NDFD calculation, whereas the dNDF calculation uses the NDF determination only once. However, Spanghero *et al.* (2010) and Hall & Mertens (2012) could not prove this theory. When determining NDFD, taking isolated NDF as a sample instead of whole forage as a sample may improve precision (Deaville & Givens, 2001).

The fermentation step with rumen fluid is the main cause of variation in the *in vitro* measurement of NDFD. In the standard *in vitro* technique, each sample is incubated with rumen fluid in separate tubes. Residue NDF analysis is performed after transferring fluids from the tubes to a beaker. This transferring step is not necessary when filter bags are used. However, filter bags may limit forage sample surface area available to rumen microbes and the microenvironment within bags can differ from the environment of the incubation medium. The Daisy^{II} incubator (Ankom Technology, Fairport, NY) is a filter bag *in vitro* system to measure NDFD in a fast and simple way: forage samples are enclosed in filter bags and rotated within glass jars filled with rumen fluid. The technique gives acceptable digestibility estimates when the emphasis is on saving labor, but it is less precise than the standard method (Wilman & Adesogan, 2000). Digestibility results obtained by the Daisy^{II} technique are affected by particle size, sample size, the proximity of the incubation jars to the heat source and the extent to which individual bags are submerged throughout the incubation (Damiran *et al.*, 2008). The sample size also influences the *in vitro* Daisy^{II} technique because losses of undigested fine particles through the pores of the bags increase when the ratio of sample size to surface area decreases. Samples within a jar can influence each other; such influence might bring samples closer to the mean digestibility of all samples within the jar.

Currently, the official Belgian variety trials report starch concentration and organic matter digestibility (OMD) as quality parameters. A given OMD value can relate to a high grain fraction and/or a high NDFD of the stover. Significant correlations between starch, NDF and OMD are described in literature, but no relationship exists between NDFD and starch/NDF (Barrière *et al.*, 2003; Hetta *et al.*, 2012). To include NDFD as a quality parameter in the variety trials report, it must provide additional information about the energy source of the plant.

Hypothesis **H1b: Measurements of NDFD are suitable for routine evaluation of the nutritive value** is studied by answering following research questions: (**RQ4**) "What is the best *in vitro* method to determine NDFD?" and (**RQ5**) "Can NDFD be estimated based on NDF/starch and OMD?" (Figure 3.2).

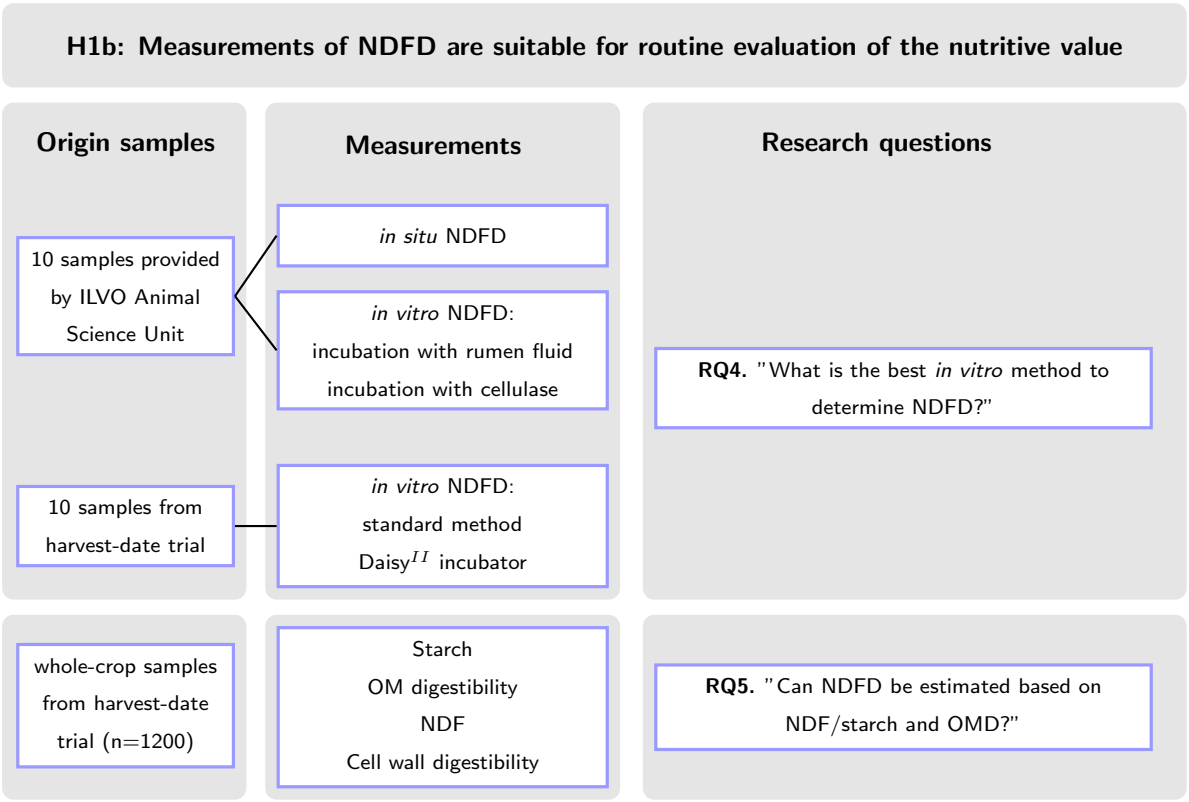


Figure 3.2: Schematic presentation of the research linked to H1b. All measurements were applied to all samples, except for the relations indicated by a line. When a line is shown, performances were limited to the relations indicated by a line

3.2.2 Materials and methods

In situ NDFD

ILVO animal Science Unit provided a selected set of 10 forage maize samples from their collection. These samples had a wide range of *in situ* NDF fermentability and were used as references to test the *in vitro* methods. The *in situ* NDF fermentability was determined with the nylon bag technique (CVB, 2004) using three rumen cannulated cows. Nylon bags (Sefar, Heiden, Switzerland) measuring 8x10 cm and with a pore size of 37 μm were filled with 2.5 or 5 g DM-equivalent of the feed and were then heat-sealed. Three bags per cow were incubated in the rumen during 8, 24, 48, 72 and 336 h. Besides, 3 bags, filled with sample, were not incubated in the rumen, but underwent all other treatments to determine the washout fraction. After incubation, bags with residues were immediately immersed in ice water, further rinsed under running tap water and put in a freezer (-18 °C). After collection of all bags, they were machine-washed (Zanussi, Frankfurt/Main, Germany) with cold water without spin cycle and then freeze-dried. Residues from the three cows were pooled per incubation time and ground to pass a 1-mm screen (Retsch ZM-1) for analysis of NDF.

In vitro NDFD

An overview of the *in vitro* NDFD methods is given in Table 3.7. Our standard *in vitro* NDFD method is explained in detail in Chapter 2: General materials and methods. This *in vitro* NDFD method was performed with the set of 10 samples from ILVO Animal Unit with known *in situ* NDF fermentability (set1) and a set of 5 samples from the harvest-date trial (set2), comparing incubation times of 24 h and 48 h. The experimental design of the harvest-date trial can be found in Chapter 2. Further, an enzymatic *in vitro* method was tested on the first set of samples. With the enzymatic *in vitro* method, an incubation with cellulase (from *Trichoderma viride*) for 24 and 48 h is performed on pre-extracted NDF. The Daisy^{II} incubator was tested with the second set of samples. With the Daisy^{II} incubator, forage samples (0.5 g) were enclosed in filter bags (Ankom F57) and rotated within glass jars filled with rumen fluid at 39 °C for 48 h. After incubation, the bags were removed from the jars and rinsed thoroughly with cold water and immediately analysed for NDF using the Ankom Fiber Analyzer. All measurements were performed during 2 runs, set1 in duplicate and set2 in triplicate.

Table 3.7: Overview of *in vitro* NDFD methods

Method	Inoculum	Technique	Incubaton time	Sample
	Rumen fluid	Separate tubes	24 h	Set 1 (n=10) and 2 (n=5)
Our standard method	Rumen fluid	Separate tubes	48 h	Set 1 (n=10) and 2 (n=5)
Enzymatic method	Cellulase	Separate tubes	24 h	Set 1 (n=10)
Enzymatic method	Cellulase	Separate tubes	48 h	Set 1 (n=10)
Daisy^{II} method	Rumen fluid	Daisy^{II} incubator	48 h	Set 2 (n=5)

Calculations

Repeatability and reproducibility were calculated using the values of set2. Triplicate measurements (k=3) at 2 fermentation runs (batch α,i=2) obtained from 5 samples (β,j=5), were analysed using the following linear model (μ=overall mean; ϵ=residual error):

$Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \epsilon_{ijk}$

The variance components of batch effect, its interaction with the sample effect and the error variance (σ_B^2, σ_i^2, σ_e^2, respectively) were used to calculate the standard deviation (SD) of repeatability (equation 3.4) and reproducibility (equation 3.5).

$$\text{Repeatability} = \sqrt{\sigma_e^2} \tag{3.4}$$

$$\text{Reproducibility} = \sqrt{\sigma_e^2 + \frac{\sigma_i^2 - \sigma_e^2}{k} + \frac{\sigma_B^2 - \sigma_i^2}{jk}} \tag{3.5}$$

Repeatability and reproducibility were then expressed as coefficients of variation (SD/mean x 100), which were used as precision terms.

The *in vitro* NDFD can be calculated assuming that the non-NDF part of plant material is completely digestible (Barrière *et al.*, 2003): NDFD = 1000 x (OMD - (1000-NDF))/NDF. Furthermore, NDF is negatively related to starch (Barrière *et al.*, 2003), so NDFD can also be calculated from starch and OMD by substituting NDF by (1000-starch). Following equations were compared with the measured NDFD values (the standard technique; incubation with rumen fluid for 48 h) using the data from the harvest-date trial (whole-crop results, n=1200):

$$\text{NDFD} = 1000 \times (OMD - (1000 - NDF))/NDF \tag{3.6}$$

$$= 1000 \times (OMD - starch)/(1000 - starch) \tag{3.7}$$

3.2.3 Results

The standard *in vitro* method underestimated *in situ* NDF fermentability after 24 h incubation while NDFD was overestimated after 48 h (Table 3.8). On average, the 24 hours longer incubation time with rumen fluid increased NDFD with 20% units. For the enzymatic *in vitro* method, increasing the incubation time with 24 hours resulted in a 6% unit mean increase in NDFD.

Table 3.8: Mean (sd) NDF, *in situ* NDF fermentability and *in vitro* NDFD: incubation with rumen fluid and cellulase; 24 and 48 h incubation

Sample (set1)	NDF (g kg^{-1}DM)	*In situ* NDF fermentability (g kg^{-1}NDF)	*In vitro* NDFD (g kg^{-1}NDF)			
			Rumen fluid		Cellulase	
			24 h	48 h	24 h	48 h
1	347	207	182 (38)	445 (32)	504 (2)	580 (29)
2	378	252	209 (11)	371 (35)	469 (7)	531 (12)
3	348	263	284 (17)	465 (35)	565 (23)	638 (24)
4	393	286	278 (28)	492 (45)	514 (21)	595 (17)
5	380	300	248 (37)	438 (28)	586 (12)	647 (11)
6	363	320	258 (37)	483 (31)	493 (2)	548 (17)
7	409	355	363 (17)	577 (28)	556 (1)	606 (8)
8	336	389	298 (8)	485 (43)	517 (9)	583 (13)
9	367	408	219 (49)	454 (28)	511 (0)	575 (19)
10	318	419	402 (20)	576 (47)	706 (9)	770 (9)

Correlations between *in situ* NDF fermentability and *in vitro* NDFD with rumen fluid were 0.56 and 0.62 for 48 h and 24 h incubation respectively (Table 3.9). Although a good relationship between *in vitro* NDFD with rumen fluid and *in vitro* NDFD with cellulase was found (r>0.6), the relationship between *in situ* NDF fermentability and *in vitro* NDFD with cellulase was weak (r<0.5).

Table 3.9: Correlations between *in situ* NDF fermentability and *in vitro* NDFD (r)

				In situ NDF fermentability	*In vitro* NDFD			
					Rumen fluid		Cellulase	
					24 h	48 h	24 h	48 h
In vitro	NDFD	Rumen fluid	24 h	0.66	-			
			48 h	0.56	0.86	-		
		Cellulase	24 h	0.46	0.76	0.66	-	
			48 h	0.38	0.73	0.63	0.99	-

A 24 hours longer incubation time improved the precision of the rumen fluid *in vitro* method (Table 3.10): repeatability decreased from 8.2 to 3.7% and reproducibility decreased from 8.6 to 4.2%. The Daisy^{II} incubator always gave greater values for NDFD compared to the standard method with 48 h incubation in separate tubes. Repeatability of the Daisy^{II} incubator was worse than the standard method with 48 h incubation, but better than a 24 h incubation in separate tubes. Reproducibility of the Daisy^{II} incubator was three times greater than its repeatability. Changing the expression of NDF degradation from NDFD to dNDF did not change repeatability and reproducibility of each tested method.

Table 3.10: Mean NDF and statistics of cell wall digestibility (expressed as NDFD and dNDF) after 24 and 48 h of incubation in separate tubes and after 48 h of incubation with the Daisy^{II} incubator. Statistics were calculated with samples 1 to 5.

		In vitro NDFD (g kg^{-1}NDF)			*In vitro* dNDF (g kg^{-1}DM)		
Sample	NDF	Separate tubes		Daisy^{II} incubator	Separate tubes		Daisy^{II} incubator
(set2)	(g kg^{-1}DM)	24 h	48 h	48 h	24 h	48 h	48 h
1	463	409	543	590	189	251	273
2	452	442	583	663	200	263	299
3	399	482	623	688	192	249	274
4	400	555	653	713	222	262	286
5	458	471	592	639	216	271	292
6	402	379	507		152	204	
7	404	328	503		133	203	
8	375	390	576		146	216	
9	452	404	593		183	268	
10	402	416	604		167	243	
Variances							
Residual error		1490	494	1928	253	90	388
Batch		11864	3812	76861	2220	764	12888
Batch x Sample		1182	557	26803	207	114	4945
Precision parameters *							
Repeatability		8.18	3.71	6.67	7.81	3.66	6.92
Reproducibility		8.85	4.17	17.68	8.58	4.22	17.32

* Coefficient of variation = SD/mean x 100

A negative relationship was found between starch and NDF (Table 3.11). Most variation in OMD is explained by variation in NDF and variation in starch. Correlations with NDFD were low for starch, OMD and NDF.

Table 3.11: Correlations (r) between starch, OMD, NDF and NDFD of the whole-crop (n=1200)

	Starch	OMD	NDF	NDFD
starch	-	0.59	-0.69	-0.16
OMD	0.59	-	-0.75	0.39
NDF	-0.69	-0.75	-	0.13
NDFD	-0.16	0.39	0.13	-

Compared to the measured value of NDFD, the calculation based on NDF had a smaller mean value and a greater standard error of the mean (3.12). The mean and standard error of the mean were similar between the calculations based on starch and the measured values.

Table 3.12: Statistics on measured and calculated NDFD (g kg^{-1}NDF)

Measured values			Calculated values	
Mean	S.E.	Calculation	Mean	S.E.
601	1.18	$1000 \times (OMD - (1000 - NDF))/NDF$	371	1.37
		$1000 \times (OMD - starch)/(1000 - starch)$	623	0.99

S.E. Standard error of the mean

Regressions of measured NDFD on the calculated values had a greater r value when calculation was based on NDF rather than on starch (Table 3.13). Taking into account DM concentration of the samples, regression equations improved to an r value of 0.743 and 0.607 for calculations based on NDF and starch respectively. Both calculations resulted in a variety rank similar to the variety rank based on the measured values.

Table 3.13: Relationships between measured and calculated NDFD (g kg^{-1}NDF)

		S.E.			Variety rank
Independent(x) variable	Equation	Intercept	Slope	r	(P value)
$1000 \times (OMD - (1000 - NDF))/NDF$	$NDFD = 382 + 0.59x$	6.8	0.018	0.700	0.099
$1000 \times (OMD - starch)/(1000 - starch)$	$NDFD = 174 + 0.69x$	17	0.027	0.603	0.051

3.2.4 Discussion

Interpretation of NDFD results in literature is difficult because many versions of the *in vitro* technique are used. Laboratory differences in rumen fluid collection procedures and type of animal donors explain the poor reproducibility between laboratories (Spanghero *et al.*, 2010). Our results with 48 h *in vitro* incubation with rumen fluid gave comparable results as obtained by Cone *et al.* (2008) and Spanghero *et al.* (2010). The disadvantages of digestibility experiments with animals can be avoided by the use of commercially available enzymatic preparations. The cellulase technique has been introduced to determine *in vitro* OMD (De Boever *et al.*, 1997). This enzymatic method is simpler to conduct and has a better reproducibility. Unfortunately, the enzymatic method is less suitable for *in vitro* NDFD evidenced by the low correlation with *in situ* NDFD. Furthermore, incubation with cellulase overestimated NDFD probably because the incubation was performed on pre-extracted NDF. Indeed, Deaville & Givens (2001) reported a greater NDFD after pre-extraction of the NDF fraction.

Spanghero *et al.* (2010) compared *in situ* NDFD values with *in vitro* NDFD values obtained with the Daisy^{II} technique and concluded that the Daisy^{II} technique was highly correlated with *in situ* data but with low repeatability and low reproducibility. Our results with the Daisy^{II} technique showed a similar repeatability and reproducibility compared to the results of Spanghero *et al.* (2010). Extending the incubation time from 24 h to 48 h improved the precision of the results because NDF degradation was more complete. Therefore, the *in vitro* method with rumen fluid incubation for 48 h will be used as a standard method in this manuscript to determine NDFD. Furthermore, changing the expression of NDF degradation as a percentage of NDF (NDFD) to a percentage of DM (dNDF) did not improve repeatability and reproducibility, in agreement with Spanghero *et al.* (2010) and Hall & Mertens (2012). Furthermore, the interpretation differs between NDFD and dNDF. When studying nutritive value, NDFD values are of interest because dNDF does not distinguish between NDF and NDFD. This means that a high dNDF can be achieved either by a high NDF, NDFD or a combination of both.

The nutritive value of forage maize is determined by the ear and the stover, providing energy in the form of starch and structural fibre respectively. As starch is almost completely digestible, most variation in OMD is explained by variation in NDF concentration and NDFD. The correlation between NDF and NDFD was close to zero, in line with Barrière *et al.* (2003) and Hetta *et al.* (2012). However, NDFD can be computed assuming that the non-NDF part of plant material is completely digestible (Barrière *et al.*, 2003). Indeed, our results (including 1200 samples at a wide range of maturation) confirmed the ability to estimate NDFD based on OMD and NDF: a good correlation was found but NDFD calculations underestimated the measured NDFD values and variability increased. We obtained a more accurate estimation (similar mean values and variability) when starch concentrations were used in the equation instead of NDF, assuming that only starch is completely digestible, but the relationship had a smaller r value compared to the regression with NDF.

Calculated NDFD values based on NDF and OMD are frequently used in literature (Lauer *et al.*, 2001; Darby & Lauer, 2002; Tagliapietra *et al.*, 2011). When the emphasis is on saving labour, NDFD estimations give acceptable values, but it is always less accurate than measuring NDFD *in vitro* or *in vivo*. Furthermore, NDFD calculations based on NDF/starch and OMD can be used as an alternative for the NDFD measurements to rank maize varieties according to NDFD as variety ranks did not differ between the measured and calculated NDFD values. The Belgian variety trials, currently reporting quality parameters starch concentration and OMD, give enough information on the energy status of the plants as NDF is closely related to starch and NDFD can be estimated from starch and OMD.

3.2.5 Conclusion

The standard *in vitro* incubation with rumen fluid for 48 h continues to be the best practice for *in vitro* NDFD determination. A weak correlation was found between *in situ* NDFD and enzymatic *in vitro* NDFD. The Daisy^{II} technique resulted in poor precision terms. In the context of variety trials, calculating NDFD based on starch concentration and OMD suffices to rank NDFD.

4

Variation in maize varieties: stay-green characterization and nutritive value

Parts of this chapter are based on: Swanckaert, J., Pannecoucque, J., Van Waes, J., Steppe, K., Van Labeke, M-C. and Reheul, D. (2016). Stay-green characterization in Belgian forage maize. *The Journal of Agricultural Science*, pp. 1-11. DOI: 10.1017/S002185961600085X

4.1 Stay-green characterization

4.1.1 Introduction

Senescence is an active phase of plant development involving degradation and remobilization processes. Stay-green (SG) is the general term given to genotypes in which senescence is delayed compared with standard reference (further referred to as "normal") genotypes (Thomas & Howarth, 2000). According to Thomas & Smart (1993) there are different types of SG. Functional SG hybrids photosynthesise longer than normal types owing to a delayed onset and/or a slower decrease in photosynthetic capacity (P_{sat}). Cosmetic SG types retain chlorophyll and chloroplast membrane proteins in senescing leaves but P_{sat} declines at a rate similar to normal types (Thomas & Howarth, 2000). Stay-green phenotypes can be the result of mutations suppressing phytohormones, alteration in the activity of transcription factors, impairment in the enzymatic steps responsible for chlorophyll breakdown, and/or alterations in metabolic pathways in chloroplasts (e.g., photosynthesis) (Kusaba *et al.*, 2013).

Functional SG types can be identified by measuring P_{sat} as the net CO_2 assimilation rate (He *et al.*, 2003; Zhang *et al.*, 2012). Measuring P_{sat} in the field is time and labour intensive. The results may be influenced by weather conditions during the measurements. Hence, most studies measure proxies to characterize the SG trait. Destructive measurements, such as chlorophyll and nitrogen (N) concentrations in the leaves suffer far less from daily weather fluctuations but they still require laboratory facilities. Leaf greenness can be measured by a SPAD portable chlorophyll meter (Chapman & Barreto, 1997; Subedi & Ma, 2005; Boussadia *et al.*, 2011) or it can simply be scored by a visual evaluation (Pommel *et al.*, 2006; Kosgey *et al.*, 2013).

Delayed leaf senescence is one of several traits that has contributed to the increased yield potential of new maize hybrids (Echarte *et al.*, 2008), but this is only valid in functional SG types. Indeed, differences in leaf senescence among older and newer forage maize hybrids have been associated with a specific difference in carbon (C) and N flux to fill grains (Rajcan & Tollenaar, 1999). Many current forage maize varieties are claimed to be SG types but it is unclear if SG in these varieties is functional or cosmetic. For example, Wilkinson & Hill (2003) found no yield advantage of SG varieties because environmental effects on yield were greater than effects of plant type. By delaying senescence, SG varieties have the opportunity to intercept more solar radiation and the potential to accumulate more dry matter (DM). A surplus of assimilates can be stored in the stover, which acts as a buffer (Rajcan & Tollenaar, 1999), or translocated to the roots, resulting in a greater post-flowering N uptake (He *et al.*, 2003). Grain N requirements during grain-filling are met from soil uptake and remobilization from the stover. However, Kosgey *et al.* (2013) reported that SG varieties had smaller grain N concentrations because they retained N in their leaves without taking up more N from the soil compared to normal varieties.

We looked for confirmation of the hypothesis **H2a: functional SG plant types can be identified by studying photosynthesis and leaf characteristics**, by answering three research questions: (**RQ6**) "Is the variation in P_{sat}, leaf N concentration, chlorophyll concentration, SPAD and greenness score between varieties large enough to define plant types?"; (**RQ7**) "How large is the effect of the SG trait on photosynthates (sucrose, furctose and starch concentration) in the leaves?" and (**RQ8**) "How large is the effect of the SG trait on N dynamics and DM yield?" (Figure 4.1).

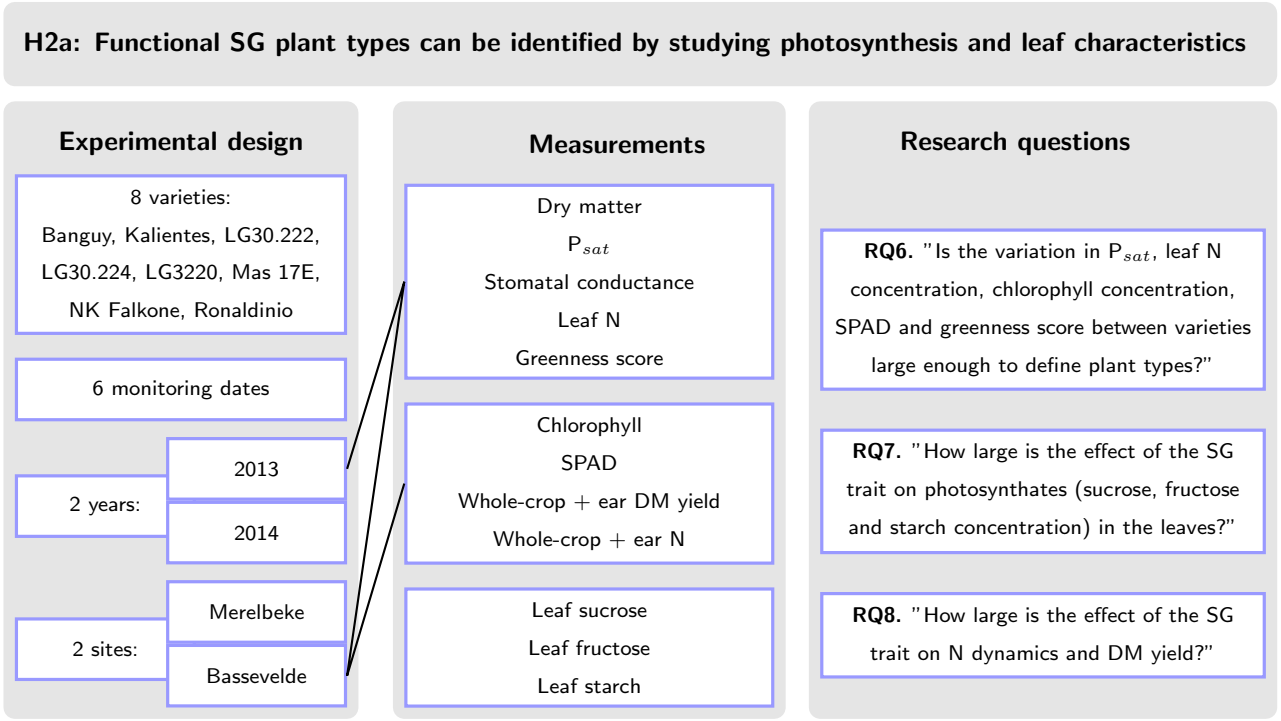


Figure 4.1: Schematic presentation of the research linked to H2a. All measurements were applied to all experimental material, except for the relations indicated by a line. When a line is shown, performances were limited to the relations indicated by a line

4.1.2 Materials and methods

Experimental design

The SG characterization is performed using the harvest-date trial (see general materials and methods, Chapter 2) in Merelbeke and Bassevelde during two consecutive years (2013-2014). The eight varieties were monitored at six monitoring dates, coinciding with the six harvest dates. We selected varieties from different companies to maximize the genetic variation between the varieties. But, the genetic background on stay-green trait was not known.

Field and laboratory measurements

Net photosynthesis at saturation level (P_{sat}) and stomatal conductance (g_s) were simultaneously measured using an open gas exchange system (LI-6400; LI-COR, Lincoln, NE, USA) with an integrated fluorescence chamber head (LI-6400-40 leaf chamber fluorometer). The light sources, air temperature

and CO_2 concentration inside the fluorescence head were set at 1500 μmol photons $m^{-2}s^{-1}$, 25 °C and 450 μmol CO_2 mol^{-1}, respectively. Measurements were made on a labelled single plant, on the third leaf from the top at the central part of the leaf blade, excluding the midrib. SPAD measurements were performed with a Konica-Minolta SPAD-502 (Konica Minolta Sensing, Inc., Osaka, Japan) on the same leaf of this plant and on the third leaf from the top of two additionally randomly chosen plants. In line with Chapman & Barreto (1997), we used the mean of three measurements near the middle of the leaf as SPAD values. The whole plot was visually scored for greenness on a scale from 1 (fully matured, brown) to 10 (green) at each monitoring date. At each monitoring date, a mixed sample of 10 cm^2 was taken from the third leaf from the top of an additional set of three randomly chosen plants and immediately taken to the laboratory. Chlorophyll was determined according to Lichtenthaler (1987). Sugars were extracted with 80% ethanol at 70 °C for 10 min and for a further three hours at 45 °C, followed by centrifugation at 5000 g for five minutes. Fructose and sucrose were analyzed using high-pH anion exchange chromatography with pulsed amperometric detection (Waters; CarboPac MA1 column with companion guard column; Thermo Fisher Benelux B.V., Amsterdam, The Netherlands; eluent: 50 mM NaOH, 22 °C). The remaining ethanol-insoluble material was washed twice with ethanol 80% and the residual pellet was treated with 1 M HCl for two hours at 95 °C for starch hydrolysis. Starch was determined spectrophotometrically at 340 nm by the enzymatic reduction of $NADP^+$ (UV-VIS, Biotek Uvikon XL, Winooski, VT, USA). At each of the monitoring dates, following plants were taken to determine DM yield, DM concentration and N concentration. A plot of 10 m^2 (approximately 100 plants) was harvested and weighted to determine fresh yield. From these 100 plants, five representative plants were randomly chosen and completely chopped and ten representative plants were used to sample leaves and ears (dehusked). The ten ears were weighted to determine fresh ear yield. Leaves and ears were chopped separately. All chopped material (whole-crop and plant parts) was dried at 70 °C for 72 hours and milled over a 1mm screen using a cutting mill (Retsch Model PK 1000). N concentration was determined by the Kjeldahl method. Measurements of P_{sat}, stomatal conductance, leaf N concentration and greenness score were performed in both years and at both sites. Chlorophyll concentration, SPAD values, DM yield and N concentration of ear and whole-crop, were only measured in 2014, at both sites. Leaf sucrose, fructose and starch concentration were only measured in Merelbeke, 2014.

Determination of measuring Ontario Units

The maturity rate (expressed as DM increase per Ontario Units (OU)) was independent of variety from monitoring date 2 to date 6. Therefore, the first monitoring date was excluded from the dataset for all following calculations. As the varieties had a different DM concentration at the onset of the measurements, we transformed monitoring dates into measuring Ontario Units (MOU) in such a way that the first MOU corresponded with a fixed DM concentration for all varieties. MOU is a measurement for plant development: it reports OU until reaching a DM concentration of 26%. MOU were calculated based on the linear regression between DM concentration and OU for variety i, year j and site k; using the following equations:

$DM_{ijk} = a_{ijk} + b_{ijk} * OU_{jk}$

$MOU_{ijk} = OU_{jk}$ - $(26 - a_{ijk})/\ b_{ijk} + 2770$

The MOU with value 2770 coincides with a DM concentration of 26%. The value of MOU at the second monitoring date is presented in Table 4.1.

Table 4.1: Measuring Ontario Units (MOU) corresponding with monitoring date 2 for each variety, year and site (MOU=2770 corresponds with 26% DM concentration)

	Merelbeke		Bassevelde	
Variety	2013	2014	2013	2014
Banguy	2863	2876	2726	2934
Kalientes	2806	3052	2741	2880
LG30.222	2796	2819	2734	2801
LG30.224	2897	2937	2702	2865
LG3220	2726	2862	2691	2836
Mas 17E	2789	2926	2703	2906
NK Falkone	2744	2857	2729	2849
Ronaldinio	2841	2831	2792	2853

Determination of photosynthetic capacity

At each monitoring date, P_{sat} of all varieties at the two sites were measured on a single day between 9 a.m. and 4 p.m. One reference plant was chosen in each field trial. P_{sat} of this reference plant was measured four times during the day to quantify potential daily changes due to changes in the environment. When the four measurements differed statistically, a linear regression was made between P_{sat} and time. This regression was used to adjust all measurements of that day. Daily P_{sat} pattern of the reference plant is presented in Appendix A.

Determination of the plant types: stay-green or normal

The eight varieties were divided into plant types based on the linear regression between P_{sat} and MOU. The full regression model included P_{sat} as dependent variable and MOU (β), type (α) with variety nested within type, year (γ; j=2013,2014), site (δ; k=Merelbeke,Bassevelde), as independent variables with all possible interactions. All factors in this model were known, except type. We calculated a set of different models: the number of models equalled the number of possible combinations of grouping the varieties; starting from 8 groups (every variety is a group) to 2 groups. The selected model with corresponding grouping of varieties fulfilled three conditions: (1) type α_i and/or its interaction with measuring date $(\alpha\beta)_i$ was/were significant; (2) effects of type α_i were independent of year γ_j and site δ_k; (3) Akaike's information criterium (AIC) (Crawley, 2007) was lowest.

4.1.3 Results

Two plant types could be identified based on P_{sat} measurements during the grain-filling period. The normal type consisted of varieties LG30.222, MAS 17E and NK Falkone. Banguy, Kalientes, LG30.224, LG3220 and Ronaldinio were characterized as SG. During the whole grain-filling period, the SG type had P_{sat} values that were 1 μmol $m^{-2}s^{-1}$ greater than corresponding values in the normal type (Figure 4.2). This difference was found in both years, even though P_{sat} decreased faster during grain filling in 2013: 4 μmol $m^{-2}s^{-1}$ per 100 MOU in 2013 compared to 3 μmol $m^{-2}s^{-1}$ per 100 MOU in 2014. The greater P_{sat} values resulted in a delayed senescence of 28 to 41 MOU, corresponding with a delayed senescence of 2 to 3 days.

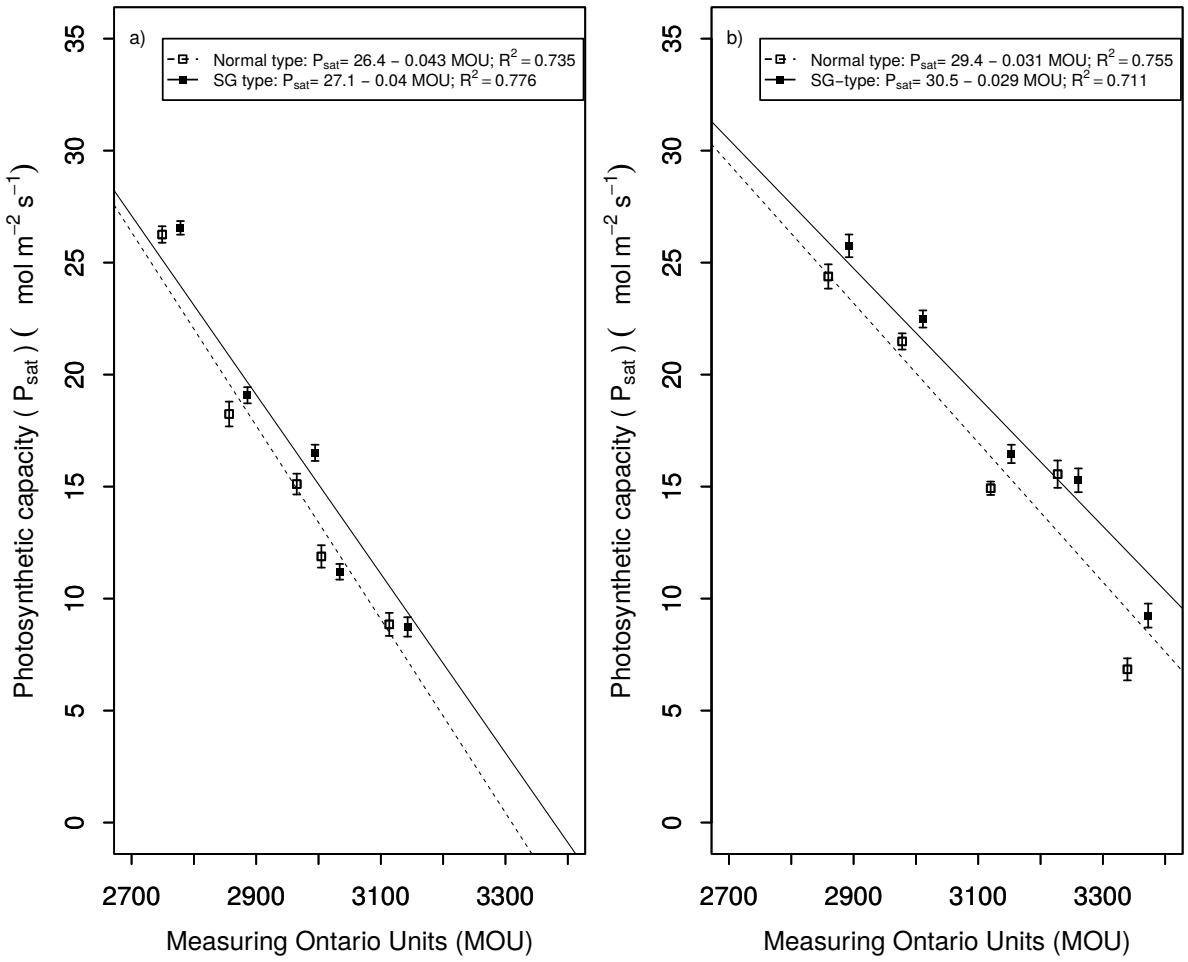


Figure 4.2: Photosynthetic capacity (P_{sat}) for the normal type (❑) and SG type (■) in (a) 2013 and (b) 2014. Each point is the mean (± S.E.) across all varieties within the type, sites Merelbeke and Bassevelde. MOU=2770 corresponds with 26% DM concentration in the harvested biomass.

The measured proxies, including leaf N concentration, chlorophyll concentration, SPAD and greenness score, were greater for the SG type compared to the normal type (Figure 4.3). These greater values were measured during the whole grain-filling period. The types were defined in such way that the effect of type on P_{sat} was independent of site, year and MOU (Table 4.2). This independence was also found with chlorophyll concentration and SPAD. The effect of type on leaf N concentration depended on year and MOU (P value = 0.002) and a significant effect of site x type interaction (P value = 0.016) was measured for greenness score. Yet, the SG varieties had a greater leaf N concentration and a greater greenness score at each site, year and MOU.

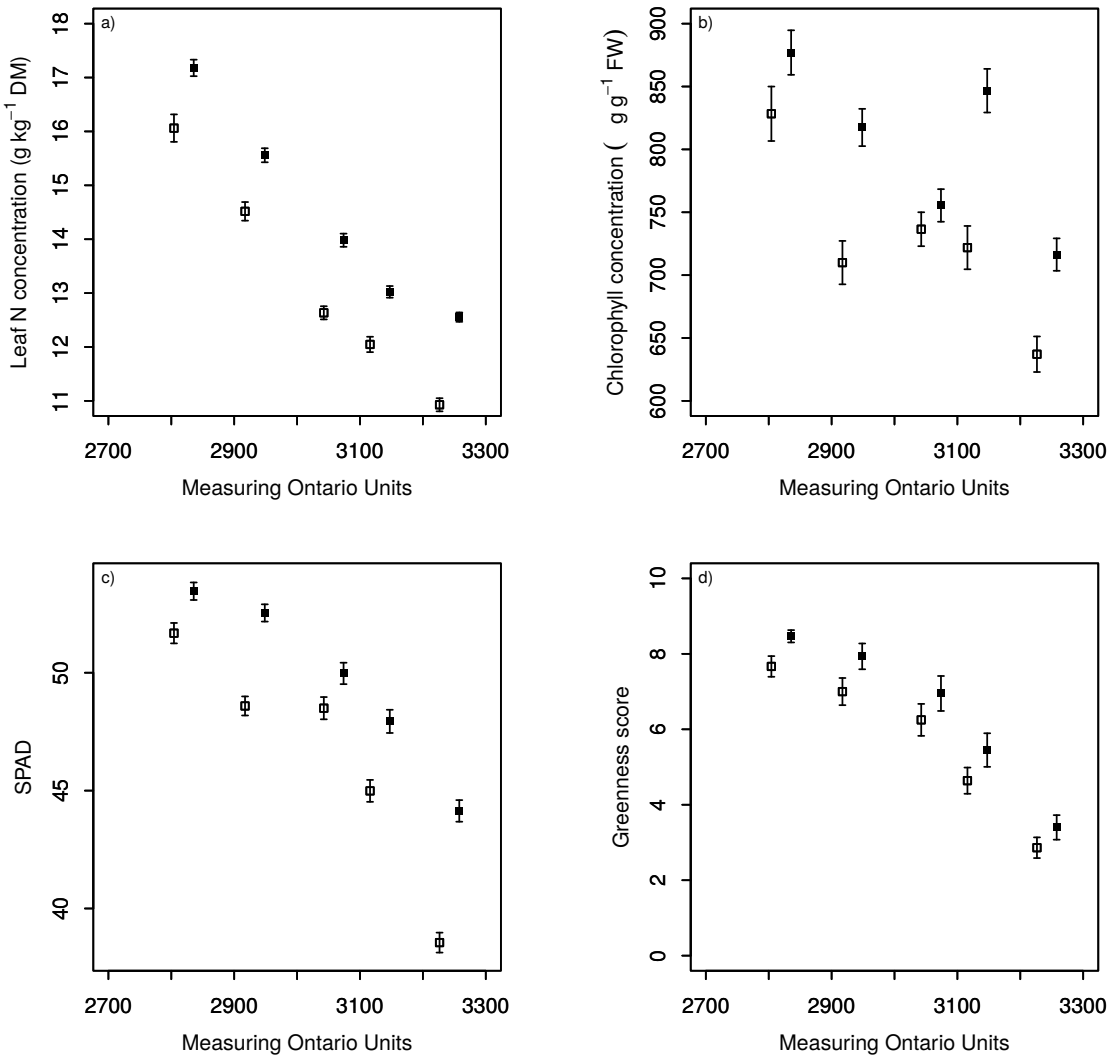


Figure 4.3: Relationship between (a) leaf N concentration, (b) chlorophyll concentration, (c) SPAD and (d) greenness score with measuring Ontario Units (MOU) for the normal type (❑) and SG type (■). Each data point is the mean (± S.E.) across all varieties within the type, sites Merelbeke and Bassevelde; years 2013 and 2014. MOU=2770 corresponds with 26% DM concentration in the harvested biomass.

Table 4.2: Evaluation of effects (expressed as P values): measuring Ontario Units (MOU), site (Site), year (Year), plant type (Type) and variety nested within plant type (Var(Type)) on photosynthetic capacity (P_{sat}), leaf N concentration, chlorophyll concentration, SPAD and greenness score.

Effect	P_{sat} (μmol $m^{-2}s^{-1}$)	Leaf N concentration (g kg^{-1}DM)	Chlorophyll concentration (μg g^{-1}FW)	SPAD	Greenness score
MOU	<0.001	<0.001	<0.001	<0.001	<0.001
Site	<0.001	0.436	<0.001	<0.001	<0.001
Year	<0.001	<0.001			<0.001
Type	<0.001	<0.001	<0.001	<0.001	<0.001
Var(Type)	0.105	<0.001	<0.001	<0.001	<0.001
MOU x Site	<0.001	<0.001	-	<0.001	<0.001
MOU x Year	<0.001	<0.001			<0.001
MOU x Type	0.85	0.313	0.69	0.184	0.13
MOU x Var(Type)	0.064	0.187	0.022	-	0.058
Site x Year	<0.001	<0.001			<0.001
Site x Type	0.922	0.023	0.168	0.539	0.016
Site x Var(Type)	0.01	<0.001	-	-	0.001
Year x Type	0.485	0.034			0.629
Year x Var(Type)	<0.001	0.008			<0.001
MOU x Site x Year	0.837	<0.001			<0.001
MOU x Site x Type	0.46	0.446	-	0.148	0.106
MOU x Site x Var(Type)	0.139	-	-	-	0.04
MOU x Year x Type	0.858	0.002			0.688
MOU x Year x Var(Type)	0.158	0.072			-
Site x Year x Type	0.64	0.047			0.358
Site x Year x Var(Type)	0.157	0.011			<0.001
MOU x Site x Year x Type	0.694	0.106			0.115
MOU x Site x Year x Var(Type)	0.035	-			-

- The parameter was excluded from the statistical model by stepwise simplification

Stomatal conductance was 0.015 mol $m^{-2}s^{-1}$ greater in SG varieties compared to normal varieties, independent of site (Table 4.3). Leaf sucrose and fructose concentration did not differ between the types. Leaf starch concentration was 0.07 g $100g^{-1}$FW greater in the normal varieties compared to the SG varieties.

Table 4.3: Mean (S.E.) stomatal conductance, sucrose concentration, fructose concentration and starch concentration in the leaves per plant type in Merelbeke, 2014 and evaluation of effects (expressed as *P* values): measuring Ontario Units (MOU), plant type (Type) and variety nested within plant type (Var(Type)).

	Stomatal conductance (mol $m^{-2}s^{-1}$)	Sucrose concentration (g $100g^{-1}$FW)	Fructose concentration (g $100g^{-1}$FW)	Starch concentration (g $100g^{-1}$FW)
Normal type	0.12	0.701	0.259	0.222
SG type	0.135	0.6	0.268	0.153
S.E.	0.0084	0.0328	0.0106	0.0086
Effect				
MOU	<0.001	-	0.208	<0.001
Type	0.012	0.105	0.598	<0.001
Var(Type)	-	<0.001	<0.001	0.052
MOU x Type	-	-	0.09	0.095
MOU x Var(Type)	-	-	-	0.008

S.E. = Standard error of the mean

- The parameter was excluded from the statistical model by stepwise simplification

Effects of plant type on whole-crop DM concentrations depended on site: SG varieties had smaller whole-crop DM concentration compared to normal varieties in Merelbeke, but the difference was absent in Bassevelde. Differences between the types in DM yield and N concentration were found in the ear (Table 4.4), but were absent in the whole-crop biomass (Table 4.5 and Figure 4.4). Normal varieties accumulated more DM in the ear than SG varieties, but at a similar rate. This resulted in an ear DM yield that was 10% greater in normal varieties than in SG varieties. The greater N retention in the leaves in SG varieties resulted in a 10% smaller ear N concentration.

Table 4.4: Evaluation of effects (expressed as P values): measuring Ontario Units (MOU), site (Site), plant type (Type) and variety nested within plant type (Var(Type)) on DM concentration, DM yield, N concentration and N export of the ear in 2014.

Effect	DM concentration (%)	DM yield (t ha^{-1})	N concentration (g kg^{-1}DM)	N export (kg ha^{-1})
MOU	<0.001	<0.001	<0.001	<0.001
Site	<0.001	<0.001	<0.001	<0.001
Type	<0.001	<0.001	<0.001	<0.001
Var(Type)	<0.001	<0.001	<0.001	<0.001
MOU x Site	0.331	-	<0.001	-
MOU x Type	0.708	-	0.159	-
MOU x Var(Type)	-	-	-	-
Site x Type	0.102	0.991	0.139	0.019
Site x Var(Type)	<0.001	0.001	-	0.001
MOU x Site x Type	0.056	-	-	-
MOU x Site x Var(Type)	-	-	-	-

- The parameter was excluded from the statistical model by stepwise simplification

Table 4.5: Evaluation of effects (expressed as P values): measuring Ontario Units (MOU), site (Site), plant type (Type) and variety nested within plant type (Var(Type)) on DM concentration, DM yield, N concentration and N export of the whole-crop in 2014.

Effect	DM concentration (%)	DM yield (t ha^{-1})	N concentration (g kg^{-1}DM)	N export (kg ha^{-1})
MOU	<0.001	<0.001	<0.001	-
Site	0.011	-	<0.001	<0.001
Type	<0.001	0.245	0.692	0.056
Var(Type)	<0.001	<0.001	<0.001	<0.001
MOU x Site	0.112	-	<0.001	-
MOU x Type	0.016	-	-	-
MOU x Var(Type)	-	-	-	-
Site x Type	<0.001	-	0.231	0.066
Site x Var(Type)	<0.001	-	<0.001	-
MOU x Site x Type	0.059	-	-	-
MOU x Site x Var(Type)	-	-	-	-

- The parameter was excluded from the statistical model by stepwise simplification

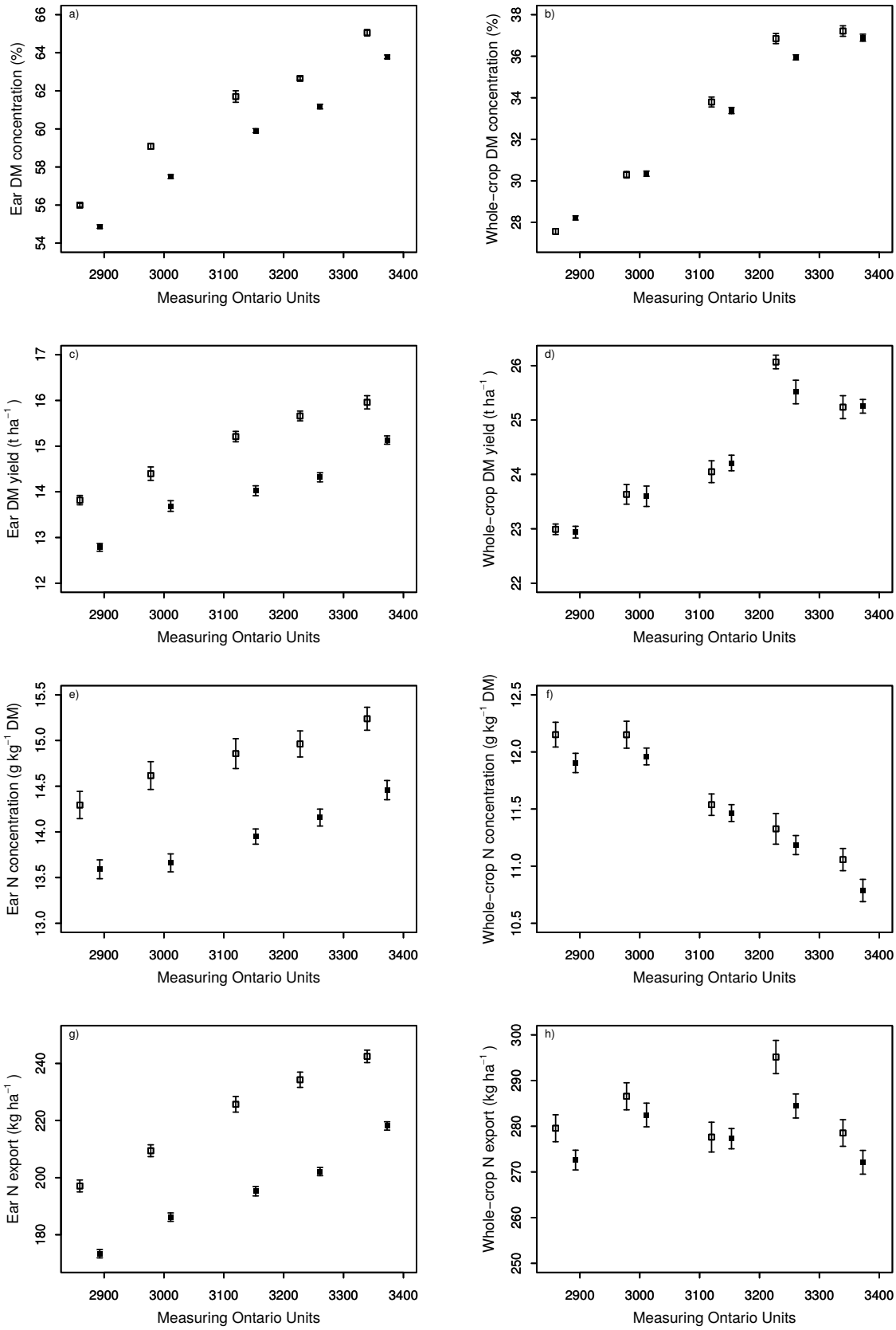


Figure 4.4: Relationship between (a) ear DM concentration, (b) whole-crop DM concentration, (c) ear DM yield, (d) whole-crop DM yield, (e) ear N concentration, (f) whole-crop N concentration, (g) ear N export and (h) whole-crop N export with measuring Ontario Units (MOU) for the normal type (❑) and SG type (■). Each data point is the mean (± S.E.) across all varieties within the type, sites Merelbeke and Bassevelde in 2014. MOU=2770 corresponds with 26% DM concentration in the harvested biomass.

4.1.4 Discussion

Variation in P_{sat} between the eight studied varieties was large enough to identify two plant types: a SG and a normal type. However by studying assimilate production and yield, we characterized the SG trait as a cosmetic one. The difference between the two plant types in P_{sat}, although significant, was probably too small to affect DM yield under the conditions of the present experiment. The use of field trials for physiological studies is challenging because environmental effects are likely to be greater than the effect of plant type. Only a few studies investigated senescence of field-grown plants by measuring P_{sat} (He *et al.*, 2003; Acciaresi *et al.*, 2014). Similar to Hirasawa & Hsiao (1999), we observed diurnal changes in P_{sat}. Therefore, a daily P_{sat} pattern of a reference plant was used to adjust all measurements. A second adjustment took into account the whole-crop DM concentration to ensure that differences in P_{sat} were caused by the SG character and not by maturation level. Values for P_{sat}, leaf N concentration and SPAD were numerically comparable with Ding *et al.* (2007) and Acciaresi *et al.* (2014). Chlorophyll concentrations reported in our study were however smaller than those reported in Ding *et al.* (2007).

The SG type showed P_{sat} values that were 1 μmol $m^{-2}s^{-1}$ greater than corresponding values in the normal type during the whole grain-filling period. Acciaresi *et al.* (2014) found significant differences in P_{sat} between normal and SG varieties only at the end of the grain-filling period. Whereas our overall difference of 1 μmol $m^{-2}s^{-1}$ was significant, the relative difference increased from 4% (26 vs 25 μmol $m^{-2}s^{-1}$) at 2700 MOU to 10% (11 vs 10 μmol $m^{-2}s^{-1}$) at 3000 MOU. SG varieties always had a greater leaf N concentration, chlorophyll concentration, SPAD and greenness score. We hypothesize that the greater chlorophyll concentration and the greater leaf N concentration were responsible for the greater P_{sat} values. Indeed, according to Osaki & Shinano (2001), a positive correlation between P_{sat} and leaf N concentration exists. Although differences in P_{sat} between SG and normal varieties were small, differences in proxies for P_{sat} were more discriminating. According to Osaki & Shinano (2001), P_{sat} is also regulated by photosynthate translocation. When photosynthates accumulate in the leaves, by low translocation of photosynthates, photosynthesis is inhibited by a feedback system (De Schepper *et al.*, 2010, 2011), which causes reduced stomatal opening (Hirasawa & Hsiao, 1999) and a smaller rate of P_{sat}. Indeed, our normal varieties showed a greater starch concentration in the leaves and a smaller stomatal conductance compared to the SG varieties, corresponding with smaller P_{sat} values. Overall, compared to normal varieties, SG varieties had greater P_{sat} values, but the difference did not result in a greater concentration of leaf photosynthates. Hence, we characterized the SG trait as a cosmetic one.

The SG trait influenced N dynamics during grain filling. Compared to normal varieties, SG varieties incorporated more N into the vegetative tissues and translocated less N from the leaves into the ears. The translocation rate (expressed as N concentration in function of MOU) was smaller for SG varieties compared to normal varieties in 2013, but not in 2014. As a result of this smaller N translocation, the ears of SG varieties contained 20 kg ha^{-1} less N than the ears of normal varieties, which corresponds to the study of Kosgey *et al.* (2013). This resulted in a 10% smaller ear N concentration in SG varieties. As C and N metabolism in kernel development is closely coupled (Cazetta *et al.*, 1999), we also found a 10% smaller ear DM yield in the SG varieties compared with the normal varieties.

Delayed leaf senescence may allow SG varieties to allocate more C and N to the roots during grain filling and maintain a greater capacity to extract N from the soil compared to normal types (Borrell *et al.*, 2001). He *et al.* (2003) compared a SG and a normal variety and observed that the former had heavier roots, richer in N. We found no evidence of greater N uptake for SG varieties in this study: whole-crop N concentration and root dry weight (data not shown) did not differ between types.

The SG trait is associated with greater moisture concentrations in the stover (leaves and stalk) (Thomas & Smart, 1993). Effects of type on whole-crop DM concentrations depended on site. Compared to normal varieties, similar whole-crop DM concentrations of SG varieties (Ettle & Schwarz, 2003; Arriola *et al.*, 2012) were only evident in Bassevelde. In contrast with Ettle & Schwarz (2003), SG varieties had a smaller ear DM concentration compared to normal varieties. Despite the similar whole-crop DM yield between the plant types, which is in line with Wilkinson & Hill (2003), the SG varieties retained a larger proportion of this total biomass in the stalk compared to the normal varieties. The limited ear DM yield of SG varieties was a result of a smaller translocation of carbohydrates, as the sink size drives the transport of carbohydrates from leaves to the developing ear (Peng *et al.*, 2014). Indeed, carbohydrate availability was not a limiting factor: water-soluble carbohydrate concentrations in the whole-crop were greater for SG varieties compared with normal varieties (based on a selection of 24 samples; data not shown).

4.1.5 Conclusion

We could identify SG varieties, but the SG trait was cosmetic. Compared to normal varieties, they showed a delayed senescence of 2 to 3 days: greater P_{sat} values, greater values for leaf N concentration, chlorophyll concentration, SPAD and greenness score. The proxies for P_{sat} were more discriminating than P_{sat} measurements. These SG varieties were characterized as cosmetic because the concentration of photosynthates (sucrose and fructose) in the leaves and whole-crop DM yield did not differ from normal varieties. The smaller P_{sat} values of the normal type coincided with an increased level of starch in the leaves and a smaller stomatal conductance. The SG trait influenced N dynamics in the plant: less translocation of N from the leaves to the ear. Compared to normal varieties, SG varieties had a smaller ear DM yield but the whole-crop DM yield did not differ between the two plant types.

4.2 Maize nutritive value

4.2.1 Introduction

Forage maize is one of the most important forage crops in ruminant nutrition in Europe, providing energy in the form of starch from the ears and structural fibre from the stover (stem and leaves). The ear is the most digestible part of the plant and accounts for about 55-65% of total dry matter (DM) yield (own data). The maize stover generally supports similar animal performances compared to average quality grass silage (O'Kiely & Moloney, 1995).

The stage of physiological development at harvest is a major factor in determining the nutritive value of forage maize. With advancing maturity and increasing DM concentrations, water-soluble carbohydrates are remobilized from the stover to the ears, where they are transformed into starch. As a result, ear development directly influences stover fibre concentrations because cell wall material accumulates. Even though there is an inverse relationship between stover quality and ear development, the whole-crop fibre concentration may decrease as a result of starch accumulation in the ear which can overcompensate for the rise in stover cell wall concentrations (Kruse *et al.*, 2008; Opsi *et al.*, 2013). These at times contradictory effects of the maturity status are the main reason why effects of maturity on whole-crop organic matter digestibility (OMD) are equivocal. Some studies have shown that OMD is constant during maturity (Hetta *et al.*, 2012; Opsi *et al.*, 2013), while others found an increase in OMD with increasing DM concentrations (Darby & Lauer, 2002; Arriola *et al.*, 2012).

In addition to the harvest time, nutritive value and digestibility of forage maize is also affected by the variety. The introduction of stay-green (SG) varieties was aimed at improving stover quality by delaying leaf senescence. The SG trait mainly provokes shifts in partition of DM yield and nitrogen (N) concentration between vegetative and generative tissues. Compared to normal varieties, SG varieties have a smaller ear and a larger stover fraction; ears contain less, stover more N (See section 2.1 and Kosgey *et al.* (2013)). Generally, SG varieties have no yield advantage because photosynthetic energy remains as sugar in the stover (Coors *et al.*, 1997). Most studies investigating the physiology of SG varieties (Thomas & Smart, 1993; Borrell *et al.*, 2001) do not link the SG trait with the nutritive value; and studies reporting effects of plant type on nutritive value do not give any physiological background of the studied varieties (Ettle & Schwarz, 2003; Cone *et al.*, 2008; Arriola *et al.*, 2012; Loucka *et al.*, 2015). As differences in nutritive value due to the SG trait depend on the variety source (company) (Arriola *et al.*, 2012) and as there is no standard method of determining the SG character of commercial varieties, generalizations about the effect of the SG trait may be misleading.

The aim of this study was to determine the influence of maturity (six harvest dates) and plant type (normal and SG) on maize nutritive value of the whole-crop and plant parts (leaves, stem and ear). We looked for confirmation of hypothesis **H2b: variation in maize nutritive value is mainly determined by maturation and SG trait**, by answering two research questions: (**RQ9**) "How does the nutritive value change during maturation?" and (**RQ10**) "How large is the effect of SG trait on maize nutritive value?" (Figure 4.5).

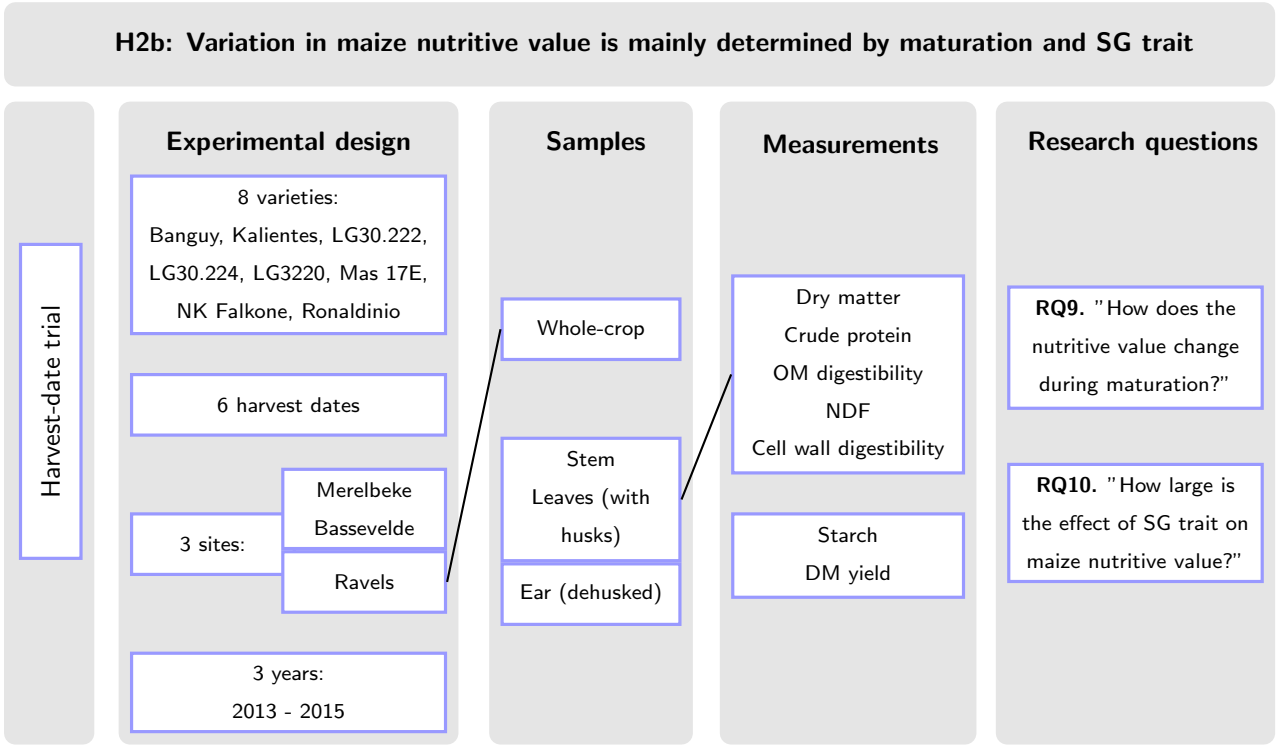


Figure 4.5: Schematic presentation of the research linked to H2b. All samples were applied to all experimental material and all measurements were applied to all samples, except for the relations indicated by a line. When a line is shown, performances were limited to the relations indicated by a line

4.2.2 Materials and methods

The harvest-date trial is used to study maize nutritive value during maturation. The experimental design, including choice of variety, harvest dates, sites and years; sampling method and determination of the maize nutritive value can be found in the chapter "general materials and methods" (Chapter 2).

4.2.3 Results

Changes in nutritive value during maturation

The results on DM concentration and maize nutritive value (crude protein (CP) concentration, starch concentration, OMD, neutral detergent fibre (NDF) and NDF digestibility (NDFD)) during grain filling are presented in Figure 4.6 and Table 4.6 for the whole-crop and plant parts. Whole-crop and ear DM concentrations increased linearly with 2% units per 100 OU. Linear models best explained the relationship between the CP concentration and OU for the whole-crop and plant parts, with the exception of the stem. For the whole-crop and the leaves, the CP concentration was greatest at earlier harvest dates and declined linearly with increasing maturities. CP concentrations in the ear increased with 1% units per 100 OU whereas CP concentrations in the stem were more or less constant. Starch concentrations in the whole-crop levelled off at 360 g kg^{-1}DM after 3000 OU, but starch concentrations in the ear continued to increase. Whole-crop OMD ranged from 730 g kg^{-1}OM at the first harvest date to 750 g kg^{-1}OM at the last harvest date. OMD of the leaves decreased quadratically. The stem and the ear had respectively the smallest (425 g kg^{-1}OM) and greatest (913 g kg^{-1}OM) values for OMD during the whole grain filling period. Whole-crop NDF varied between 380 g kg^{-1}DM and 430 g kg^{-1}DM. NDF values for stem and leaves were similar on average, but NDF in the stem decreased with 5% units per 100 OU while NDF in the leaves increased. In the ear, NDF ranged from 220 g kg^{-1}DM to 234 g kg^{-1}DM. A weak relationship was found between NDFD and OU of the whole-crop, but generally, NDFD decreased during maturation from 615 g kg^{-1}NDF to 590 g kg^{-1}NDF. A linear model best explained the relationship between NDFD and OU of the leaves and the stem: a decrease of 8 and 9.5% units per 100 OU respectively. Values for NDFD in the ear varied between 760 g kg^{-1}NDF and 780 g kg^{-1}NDF. Whole-crop and ear DM yield increased quadratically during maturation. Whole-crop DM yield reached a plateau at 3000 OU while ear DM yield continued to increase. The ear proportion (ear DM yield as a percentage of the whole-crop DM yield) increased from 54% at 2600 OU to 63% at 3200 OU. Whole-crop N export increased from 230 kg ha^{-1} to 260 kg ha^{-1}: so 30 kg ha^{-1} was extracted from the soil during this period. Ear N export increased with 70 kg ha^{-1}. This means that 40 kg N ha^{-1} was translocated from the stover to the ear.

Table 4.6: The regression equations for whole-crop, leaves, stem and ear. Data were pooled across year, site, variety and replication (whole-crop: n=216; plant parts: n=144) and regressed against Ontario Units (x) (n=6)

Parameter	Regression equation	R^2
	Whole-crop	
Dry matter (%)	$-27.9 + 0.021x$	0.873
Crude Protein (g kg^{-1}DM)	$113 - 0.013x$	0.822
Starch (g kg^{-1}DM)	$-2662 + 1.9x - 3.0 \times 10^{-4}x^2$	0.789
OM digestibility (g kg^{-1}OM)	$9482 - 9.5x + 3.4 \times 10^{-3}x^2 - 4.1 \times 10^{-7}x^3$	0.749
NDF (g kg^{-1}DM)	$-9524 + 10.7x - 3.8 \times 10^{-3}x^2 + 4.4 \times 10^{-7}x^3$	0.740
Cell wall digestibility (g kg^{-1}NDF)	$10090 - 10.1x + 3.6 \times 10^{-3}x^2 - 4.3 \times 10^{-7}x^3$	0.560
DM yield (t ha^{-1})	$6.69 + 0.0017x + 1.1 \times 10^{-6}x^2$	0.845
N export (kg ha^{-1})	$-283 + 0.31x - 4.2 \times 10^{-5}x^2$	0.786
	Leaves	
Crude protein (g kg^{-1}DM)	$263 - 0.060x$	0.855
OM digestibility (g kg^{-1}OM)	$414 + 0.25x - 3.4 \times 10^{-5}x^2$	0.799
NDF (g kg^{-1}DM)	$-2311 + 3.2x - 1.2 \times 10^{-3}x^2 + 1.5 \times 10^{-7}x^3$	0.666
Cell wall digestibility (g kg^{-1}NDF)	$947 - 0.079x$	0.800
	Stem	
Crude protein (g kg^{-1}DM)	$-1881 + 2.0x - 6.9 \times 10^{-4}x^2 + 7.8 \times 10^{-8}x^3$	0.737
OM digestibility (g kg^{-1}OM)	$3028 - 1.8x + 3.1 \times 10^{-4}x^2$	0.800
NDF (g kg^{-1}DM)	$826 - 0.053x$	0.806
Cell wall digestibility (g kg^{-1}NDF)	$817 - 0.095x$	0.886
	Ear	
Dry matter (%)	$-15.4 + 0.024x$	0.900
Crude protein (g kg^{-1}DM)	$60.6 + 0.0090x$	0.849
Starch (g kg^{-1}DM)	$-731 + 0.76x - 1.0 \times 10^{-4}x^2$	0.857
OM digestibility (g kg^{-1}OM)	$-997 + 1.8x - 6.0 \times 10^{-4}x^2 + 6.6 \times 10^{-8}x^3$	0.861
NDF (g kg^{-1}DM)	$-179 + 0.83x - 4.2 \times 10^{-4}x^2 + 6.3 \times 10^{-8}x^3$	0.778
Cell wall digestibility (g kg^{-1}NDF)	$15200 + 17.0x - 6.0 \times 10^{-3}x^2 + 7.1 \times 10^{-7}x^3$	0.761
DM yield (t ha^{-1})	$-49.1 + 0.035x - 4.8 \times 10^{-6}x^2$	0.831
N export (kg ha^{-1})	$-802 + 0.55x - 7.4 \times 10^{-5}x^2$	0.826

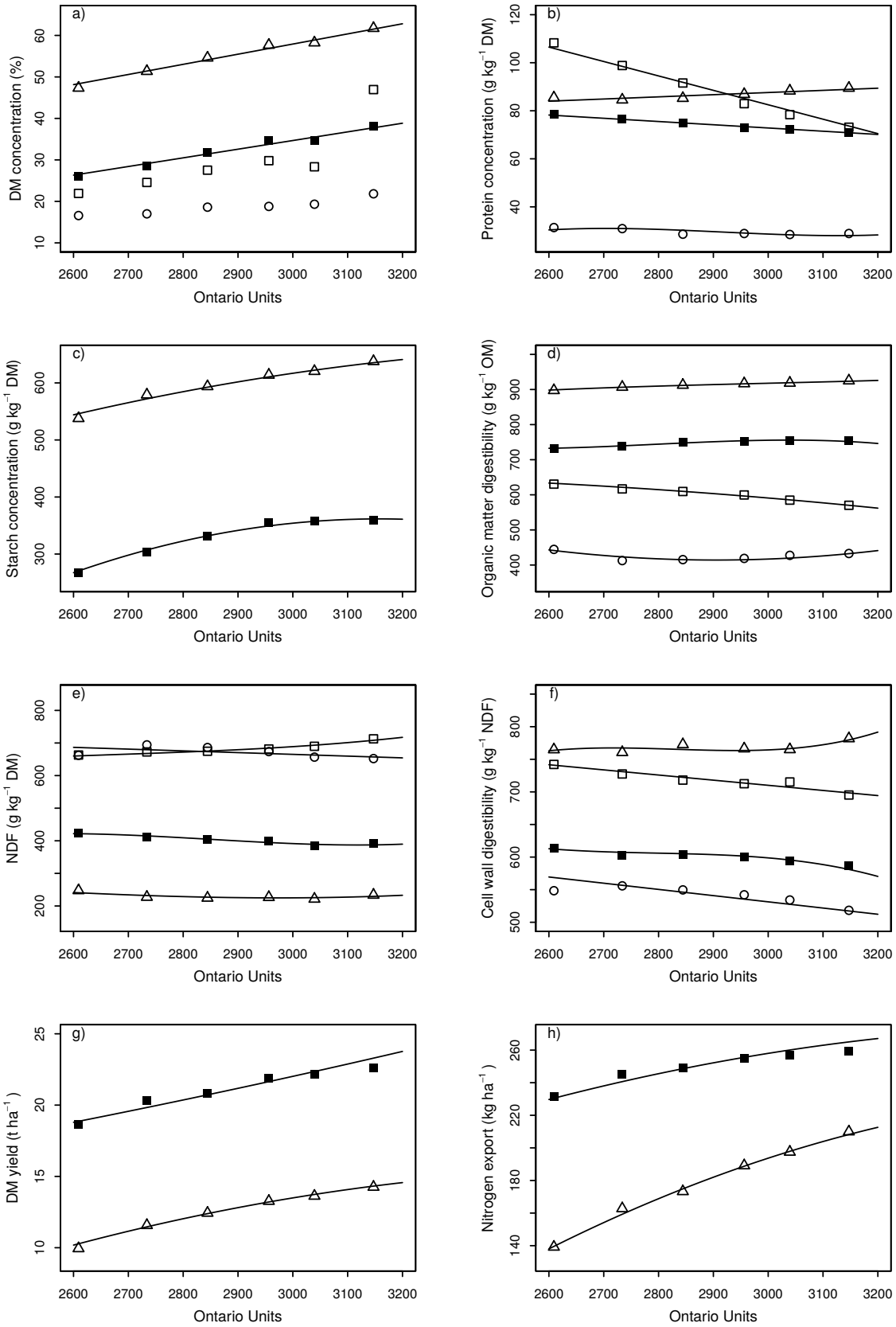


Figure 4.6: Relationship between (a) DM concentration, (b) crude protein, (c) starch, (d) organic matter digestibility (OMD), (e) NDF, (f) cell wall digestibility (NDFD), (g) DM yield and (h) nitrogen export with Ontario Units of the whole-crop (■), leaves (❑), stem (○) and ear (△). Each data point is the mean across the eight varieties, sites Merelbeke, Bassevelde and Ravels (only whole-crop) in 2013-2015.

Effect of the stay-green trait on maize nutritive value

The effect of the SG trait on whole-crop DM concentration, CP concentration, starch concentration, OMD, NDF and NDFD is presented in Figure 4.7 and Table 4.7. Although whole-crop DM concentration depended on site, the average DM concentration was always greater for the SG varieties compared to the normal varieties. As DM concentration depended on harvest date, the maturation rate (expressed as DM concentration increase per 100 OU) differed between the normal (2.3% units per 100 OU) and SG (2.0% units per 100 OU) varieties. No interactions with type were found of whole-crop CP concentration. On average, CP concentrations were 0.6 g kg^{-1}DM greater in the normal varieties compared to the SG varieties. Compared to the normal varieties, SG varieties had starch concentrations that were always greater. Dependent on the site, the difference ranged between 10 and 13 g kg^{-1}DM. The effect of SG trait on whole-crop OMD depended on the year: OMD of the SG varieties were 9 to 16 g kg^{-1}OM greater than OMD of the normal varieties. SG varieties had NDF and NDFD values that were 13 g kg^{-1}DM smaller and 14 g kg^{-1}NDF greater than normal varieties, respectively.

Table 4.7: Evaluation of effects (expressed as P values): harvest date (HD), site (Site), year (Year), plant type (Type) and variety nested within plant type (Var(Type)) on dry matter (DM), crude protein (CP), starch, organic matter digestibility (OMD), NDF and cell wall digestibility (NDFD) of the whole-crop.

Effect	DM concentration (%)	CP concentration (g kg^{-1}DM)	Starch concentration (g kg^{-1}DM)	OMD (g kg^{-1}OM)	NDF concentration (g kg^{-1}DM)	NDFD (g kg^{-1}NDF)
HD	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Type	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Site	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Year	0.013	< 0.001	0.001	< 0.001	< 0.001	0.511
HD x Type	< 0.001	-	0.248	0.573	-	-
Var(Type)	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
HD x Site	< 0.001	0.477	< 0.001	0.025	< 0.001	< 0.001
Type x Site	< 0.001	0.363	0.032	0.415	-	-
HD x Year	< 0.001	< 0.001	< 0.001	0.475	0.117	0.001
Type x Year	0.621	0.459	0.766	0.622	-	-
Site x Year	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
HD x Var(Type)	0.039	-	< 0.001	-	-	-
HD x Type x Site	-	-	0.766	-	-	-
Site x Var(Type)	< 0.001	0.581	0.008	0.3	-	-
HD x Type x Year	-	-	0.744	0.036	-	-
Year x Var(Type)	0.001	0.019	0.027	0.005	-	-
HD x Site x Year	0.001	0.005	0.098	< 0.001	-	< 0.001
Type x Site x Year	0.59	0.186	0.758	0.168	-	-
HD x Var(Type) x Site	-	-	-	-	-	-
HD x Var(Type) x Year	-	-	-	-	-	-
HD x Type x Site x Year	-	-	0.138	-	-	-
Var(Type) x Site x Year	< 0.001	0.009	0.023	0.01	-	-
HD x Var(Type) x Site x Year	-	-	-	-	-	-

- The parameter was excluded from the statistical model by stepwise simplification

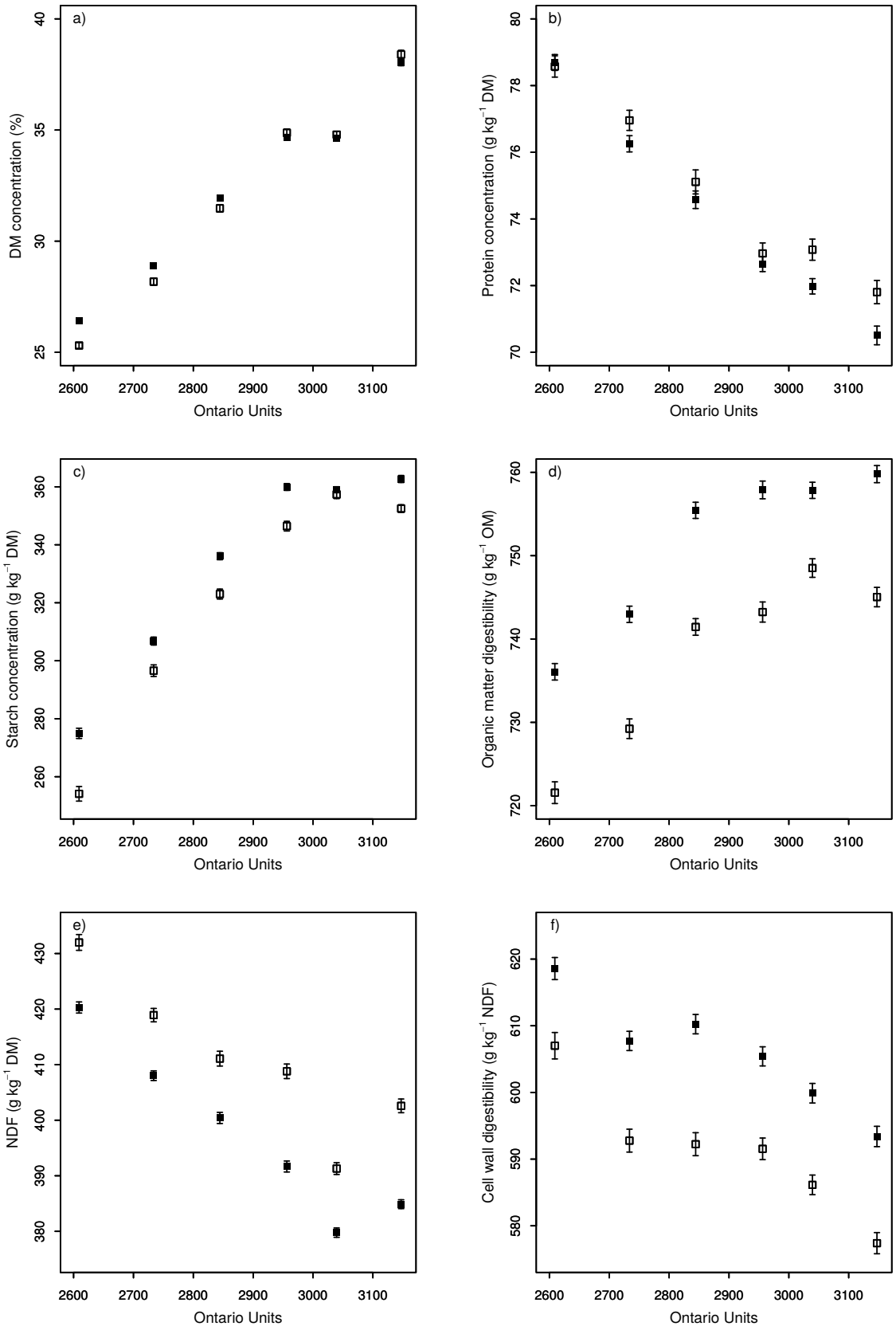


Figure 4.7: Relationship between (a) dry matter, (b) crude protein, (c) starch, (d) organic matter digestibility (OMD), (e) NDF and (f) cell wall digestibility (NDFD) with Ontario Units for whole-crop of the normal type (❑) and SG type (■). Each data point is the mean (± S.E.) across varieties within the type, sites Merelbeke, Bassevelde and Ravels in 2013-2015.

The effect of the SG trait on CP concentration in the leaves depended on site and year (Figure 4.8 and Table 4.8). Yet, at each field trial, values of CP concentrations for SG varieties were always greater (between 0.4 and 11 g kg^{-1}OM) than corresponding values for normal varieties. Compared to normal varieties, SG varieties had greater values of OMD in the leaves, but the differences depended on year and were greater at the end of the harvesting period. NDF of SG varieties were 13 g kg^{-1}DM smaller in SG varieties compared to normal varieties. The SG trait only had an effect on NDFD in 2013 and 2015 with greater values for the SG varieties compared to the normal varieties.

Table 4.8: Evaluation of effects (expressed as P values): harvest date (HD), site (Site), year (Year), plant type (Type) and variety nested within plant type (Var(Type)) on crude protein (CP), organic matter digestibility (OMD), NDF and cell wall digestibility (NDFD) of the leaves.

Effect	CP concentration (g kg^{-1}DM)	OMD (g kg^{-1}OM)	NDF concentration (g kg^{-1}DM)	NDFD (g kg^{-1}NDF)
HD	< 0.001	< 0.001	< 0.001	< 0.001
Type	< 0.001	< 0.001	< 0.001	0.018
Site	< 0.001	0.016	0.943	0.014
Year	< 0.001	< 0.001	< 0.001	< 0.001
HD x Type	0.001	< 0.001	0.059	-
Var(Type)	< 0.001	< 0.001	< 0.001	< 0.001
HD x Site	< 0.001	< 0.001	< 0.001	0.32
Type x Site	< 0.001	0.086	-	0.085
HD x Year	< 0.001	< 0.001	< 0.001	0.002
Type x Year	< 0.001	0.003	0.656	< 0.001
Site x Year	< 0.001	0.001	< 0.001	< 0.001
HD x Var(Type)	0.069	-	-	-
HD x Type x Site	0.704	-	-	-
Site x Var(Type)	0.689	-	-	-
HD x Type x Year	0.002	-	-	-
Year x Var(Type)	< 0.001	< 0.001	0.001	< 0.001
HD x Site x Year	< 0.001	0.048	0.011	< 0.001
Type x Site x Year	0.004	0.052	-	-
HD x Var(Type) x Site	0.042	-	-	-
HD x Var(Type) x Year	-	-	-	-
HD x Type x Site x Year	-	-	-	-
Var(Type) x Site x Year	0.031	-	-	-
HD x Var(Type) x Site x Year	-	-	-	-

- The parameter was excluded from the statistical model by stepwise simplification

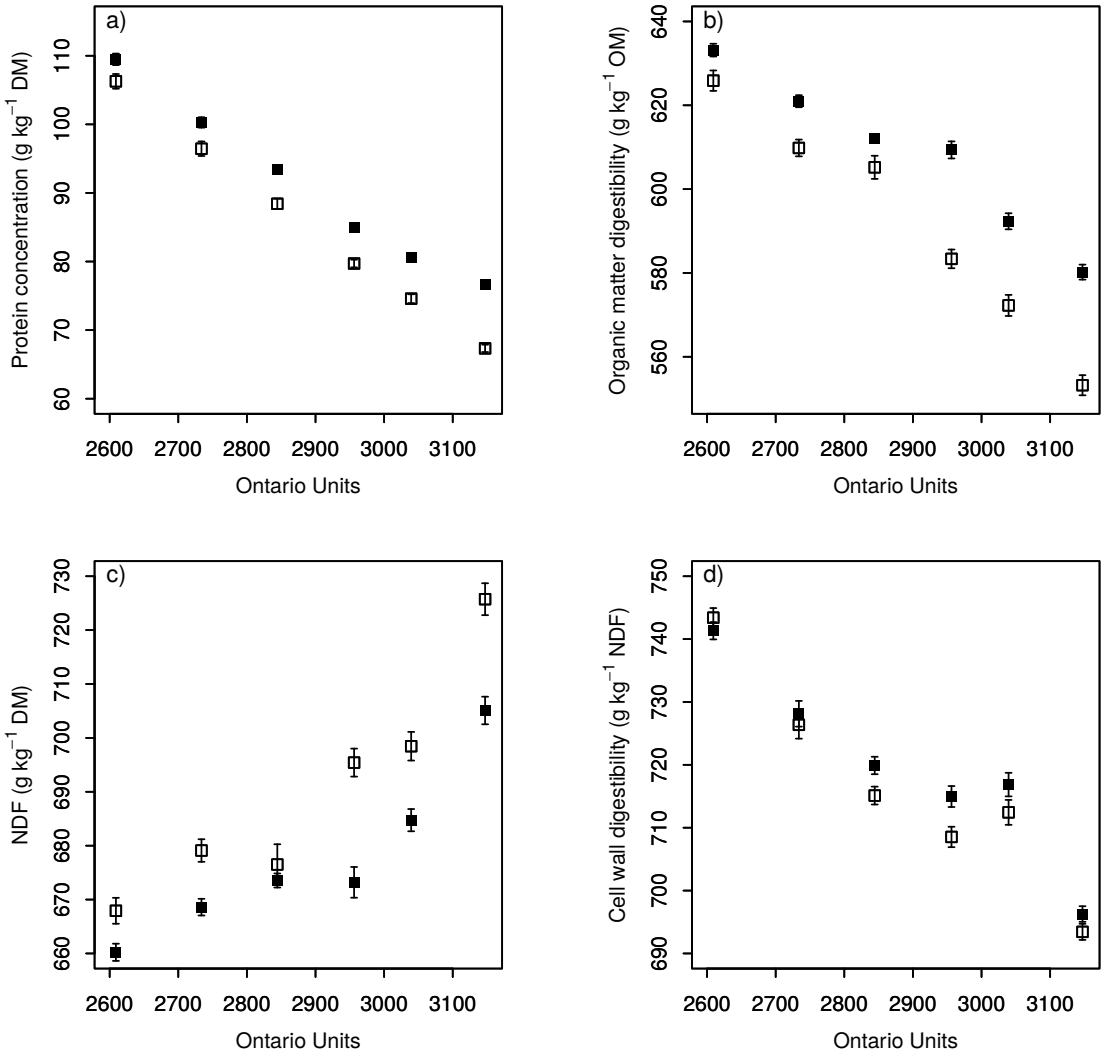


Figure 4.8: Relationship between (a) crude protein, (b) organic matter digestibility (OMD), (c) NDF and (d) cell wall digestibility (NDFD) with Ontario Units for leaves of the normal type (❑) and SG type (■). Each data point is the mean (± S.E.) across varieties within the type, sites Merelbeke and Bassevelde in 2013-2015.

The effect of SG trait on nutritive value of the stem are presented in Figure 4.9 and Table 4.9. Differences in CP concentration between SG and normal varieties depended on site and year: ranging from 0.1 to 2 g kg^{-1}DM. Compared to normal varieties, SG varieties had OMD values that were 31 to 34 g kg^{-1}OM greater dependent on site. Values for NDF were 27 g kg^{-1}DM greater for the normal varieties compared to the SG varieties. Dependent on site and year, NDFD was 3 to 24 g kg^{-1}NDF greater for SG varieties compared to normal varieties.

Table 4.9: Evaluation of effects (expressed as P values): harvest date (HD), site (Site), year (Year), plant type (Type) and variety nested within plant type (Var(Type)) on crude protein (CP), organic matter digestibility (OMD), NDF and cell wall digestibility (NDFD) of the stem.

Effect	CP concentration (g kg^{-1}DM)	OMD (g kg^{-1}OM)	NDF concentration (g kg^{-1}DM)	NDFD (g kg^{-1}NDF)
HD	< 0.001	0.95	< 0.001	< 0.001
Type	< 0.001	< 0.001	< 0.001	< 0.001
Site	0.525	< 0.001	< 0.001	< 0.001
Year	< 0.001	< 0.001	< 0.001	< 0.001
HD x Type	0.132	0.962	0.698	0.133
Var(Type)	< 0.001	< 0.001	< 0.001	< 0.001
HD x Site	< 0.001	< 0.001	< 0.001	< 0.001
Type x Site	< 0.001	0.661	0.141	< 0.001
HD x Year	0.397	< 0.001	< 0.001	0.049
Type x Year	0.041	0.821	0.989	0.889
Site x Year	< 0.001	< 0.001	< 0.001	< 0.001
HD x Var(Type)	0.004	< 0.001	< 0.001	-
HD x Type x Site	0.266	0.031	0.108	0.038
Site x Var(Type)	-	0.005	0.003	< 0.001
HD x Type x Year	0.257	-	-	-
Year x Var(Type)	< 0.001	< 0.001	< 0.001	< 0.001
HD x Site x Year	< 0.001	< 0.001	0.019	< 0.001
Type x Site x Year	< 0.001	0.173	-	0.034
HD x Var(Type) x Site	-	-	-	-
HD x Var(Type) x Year	-	-	-	-
HD x Type x Site x Year	0.051	-	-	-
Var(Type) x Site x Year	-	0.001	-	< 0.001
HD x Var(Type) x Site x Year	-	-	-	-

- The parameter was excluded from the statistical model by stepwise simplification

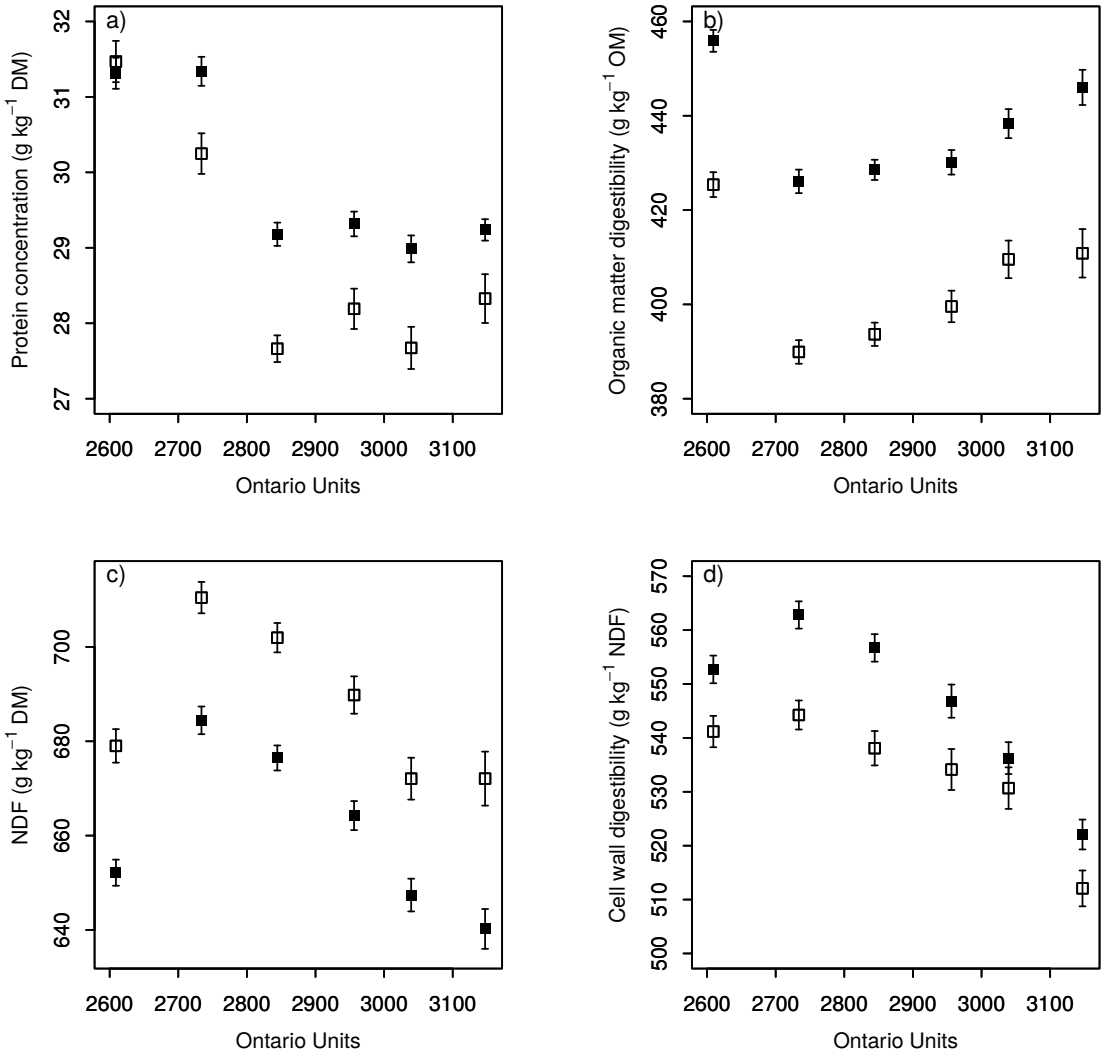


Figure 4.9: Relationship between (a) crude protein, (b) organic matter digestibility (OMD), (c) NDF and (d) cell wall digestibility (NDFD) with Ontario Units for the stem of the normal type (❑) and SG type (■). Each data point is the mean (± S.E.) across varieties within the type, sites Merelbeke and Bassevelde in 2013-2015.

The effect of SG trait on ear DM concentration, CP concentration, starch concentration, OMD, NDF and NDFD is presented in Figure 4.10 and Table 4.10. As DM concentration depended on harvest date, the maturation rate (expressed as DM concentration increase per 100 OU) differed between the normal (2.6% units per 100 OU) and SG (2.4% units per 100 OU) varieties. CP concentrations were on average 5 g kg^{-1}DM greater for normal varieties compared to SG varieties. The effect of SG trait on starch concentration depended on harvest date: the starch concentrations increased with 19 g kg^{-1}DM per 100 OU in the normal varieties compared with 15 g kg^{-1}DM per 100 OU in the SG varieties. Compared to normal varieties, SG varieties had OMD values that were greater at harvest dates 1 and 2, equal at harvest dates 3 to 5 and smaller at harvest date 6. SG varieties had NDF values that were smaller at harvest date 1, equal at harvest date 2 to 5 and greater at harvest date 6 compared to normal varieties. NDFD in the ear was 7 and 6 g kg^{-1}NDF greater for SG varieties compared to normal varieties in Merelbeke and Bassevelde respectively.

Table 4.10: Evaluation of effects (expressed as P values): harvest date (HD), site (Site), year (Year), plant type (Type) and variety nested within plant type (Var(Type)) on dry matter (DM), crude protein (CP), starch, organic matter digestibility (OMD), NDF and cell wall digestibility (NDFD) of the ear.

Effect	DM concentration (%)	CP concentration (g kg^{-1}DM)	Starch concentration (g kg^{-1}DM)	OMD (g kg^{-1}OM)	NDF concentration (g kg^{-1}DM)	NDFD (g kg^{-1}NDF)
HD	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Type	0.026	< 0.001	0.007	0.323	0.458	< 0.001
Site	< 0.001	< 0.001	< 0.001	< 0.001	0.252	0.27
Year	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
HD x Type	0.006	0.274	< 0.001	0.005	0.003	0.365
Var(Type)	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
HD x Site	< 0.001	< 0.001	0.048	0.001	< 0.001	0.005
Type x Site	-	0.062	0.549	-	0.66	0.767
HD x Year	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Type x Year	< 0.001	0.1	0.011	0.067	0.73	0.166
Site x Year	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
HD x Var(Type)	0.012	-	0.001	-	0.591	0.004
HD x Type x Site	-	0.08	0.068	-	0.286	0.012
Site x Var(Type)	-	< 0.001	0.013	-	0.636	-
HD x Type x Year	-	-	-	-	-	-
Year x Var(Type)	< 0.001	< 0.001	-	-	0.003	< 0.001
HD x Site x Year	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Type x Site x Year	-	0.001	-	-	-	0.077
HD x Var(Type) x Site	-	-	-	-	0.07	-
HD x Var(Type) x Year	-	-	-	-	-	-
HD x Type x Site x Year	-	-	-	-	-	-
Var(Type) x Site x Year	-	0.002	-	-	-	-
HD x Var(Type) x Site x Year	-	-	-	-	-	-

- The parameter was excluded from the statistical model by stepwise simplification

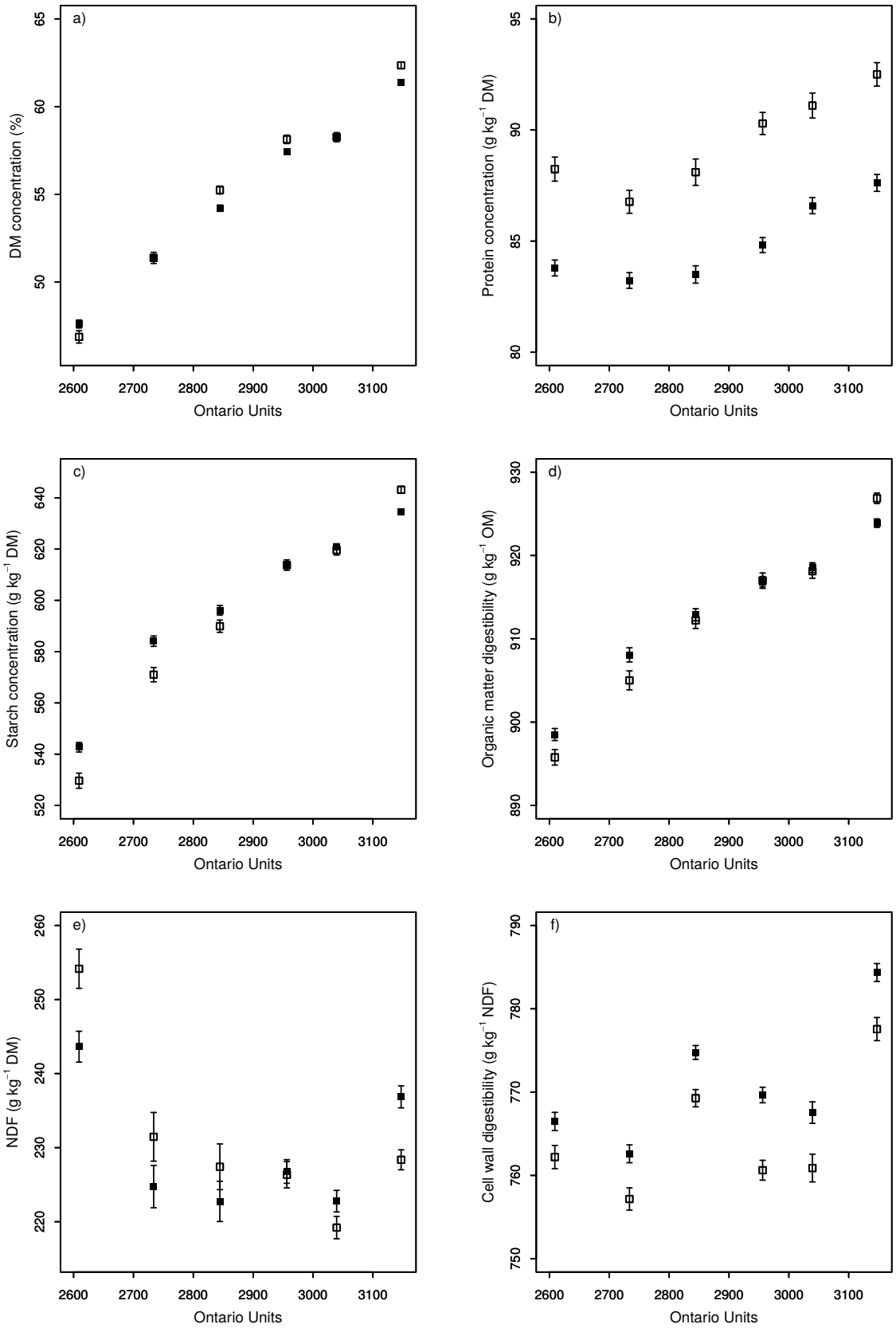


Figure 4.10: Relationship between (a) dry matter concentration, (b) crude protein, (c) starch, (d) organic matter digestibility (OMD), (e) NDF and (f) cell wall digestibility (NDFD) with Ontario Units for the ear of the normal type (❑) and SG type (■). Each data point is the mean (± S.E.) across varieties within the type, sites Merelbeke and Bassevelde in 2013-2015.

4.2.4 Discussion

The current trials were conducted with a limited set of eight varieties. These eight varieties differed in earliness (the DM concentration differed with maximum 3% units at any harvest date), energy source (cell walls or starch) and SG trait (three normal varieties and five SG varieties). Changes in nutritive value during maturation were numerically comparable with Hetta *et al.* (2012), who studied three maize varieties in Sweden at four harvest dates and determined nutritive value of the whole-crop, leaves, stem and ear. Whole-crop DM and starch concentrations increased with maturity, whereas CP concentrations decreased linearly. Maize has a low protein concentration of about 75 g kg^{-1}DM as a C4 photosynthesis plant. Consequently, the N reserve in the leaves is not sufficient to supply the growing ear, and for this reason maize is dependent on N taken up by the roots during grain filling. Indeed, an extra 30 kg N ha^{-1} is exported by the whole-crop during the studied harvesting period. In the present study, OMD decreased after reaching a maximum at 3000 OU, in line with Darby & Lauer (2002) and Arriola *et al.* (2012). Other studies reported OMD values that were unaffected by the maturity stage (Ettle & Schwarz, 2003; Opsi *et al.*, 2013). Whole-crop NDF decreased while an increase in NDF in the leaves was measured, in agreement with Kruse *et al.* (2008) and Opsi *et al.* (2013). The decrease in whole-crop NDFD during maturation is consistent with results of Hetta *et al.* (2012). The effect of maturity on the reduction of NDFD is most likely due to increased cell wall thickness, as suggested by Boon *et al.* (2008).

The effect of SG trait on maize nutritive value has been studied previously (Ettle & Schwarz, 2003; Cone *et al.*, 2008; Arriola *et al.*, 2012; Loucka *et al.*, 2015), but these studies lack a description of the SG varieties used in their trials. We measured photosynthetic capacity and N dynamics of the eight varieties to statistically support the SG characterization (See section 2.1). SG can be viewed as a consequence of the balance between N demand by the grain and N supply by translocation or extraction from the soil (Borrell *et al.*, 2001). As CP concentration is calculated by multiplying total N by 6.25; CP concentrations are expected to be influenced most by the SG trait. Both leaves and stem of SG varieties had greater CP concentrations than normal varieties. These results confirm the SG classification and are also found in the results reported by Cone *et al.* (2008) and Arriola *et al.* (2012). However, Ettle & Schwarz (2003) reported smaller CP concentrations in the stover of the SG variety compared to a variety with a fast maturing stover, which questions the reliability of the chosen varieties in their study. Compared to normal varieties, SG varieties had greater OMD values for the whole-crop and each plant part. Published results on effects of SG on OMD are equivocal. Arriola *et al.* (2012) and Cone *et al.* (2008) reported similar OMD results for SG and normal varieties, but Ettle & Schwarz (2003) associated the SG trait with smaller OMD. In SG varieties, assimilates are stored as sugar in the stover, thus diluting the fibre content. Indeed, NDF concentrations were smaller for the SG varieties compared to normal varieties. In contrast, Loucka *et al.* (2015) reported a greater NDF in SG varieties compared to normal varieties.

We found differences in nutritive value between plant types in both whole-crop and stover, but these differences were more pronounced in the stem. OMD of the stem was highly variable and associated with a large variation in NDF and NDFD. Thus, an improvement in stem OMD would present an opportunity to increase the nutritive value of the total forage maize, when starch concentrations remain unchanged.

4.2.5 Conclusion

Harvesting forage maize at high DM concentration maximized DM yield, starch accumulation and OMD, whereas NDF and NDFD decreased. CP concentration decreased during maturation while whole-crop yield increased. Because N was extracted from the soil during grain filling, N export increased. The SG trait had a positive effect on maize nutritive value. During the whole grain-filling period, SG varieties had a greater starch concentration, greater OMD, smaller NDF and greater NDFD in the whole-crop and stover. Differences in nutritive value between the plant types were more pronounced in the stem.

5

Harvest window

Parts of this chapter are based on: Swanckaert, J., Pannecoucque, J., Van Waes, J., De Cauwer, B., Latré, J., Haesaert, G., and Reheul, D. (2016). Harvest date does not influence variety ranking in Belgian forage maize variety trials. *The Journal of Agricultural Science*, 154, 1040-1050.

5.1 Introduction

Official variety trials compare agronomic performance of new varieties with reference varieties. For silage maize, Belgian evaluation criteria include parameters for dry matter (DM) yield, harvest security (resistance to lodging and stalk rot), disease resistance and quality (Van Waes, 2009). Currently, varieties are tested in one trial network covering six locations situated in the important silage-maize-growing areas in Belgium. Trials are conducted in randomized block designs for at least 2 years. Owing to the relatively small variation in growing and weather/environmental conditions in the maize-growing areas, varieties submitted for registration in Belgium show a limited variation in earliness. All varieties are harvested on one day per location, based on a whole-crop DM concentration of approximately 34% of a reference variety (with an average maturity type). Upon data processing, varieties are grouped into early and a late maturity groups. Each variety is then assigned to one of the groups based on the statistical analysis of whole-crop DM concentration at harvest. This methodology has been criticized. An alternative has been suggested, with several harvesting dates per location. This approach is based on the idea that varieties should be harvested at the physiological stage where they can show their optimal performance. Harvesting at a time with potentially sub-optimal performance is therefore expected to result in inconsistent variety ranking.

The nutritive value of maize silage is mostly affected by the choice of variety and the stage of physiological development at harvest. The effect of increased DM concentration on animal performance is well known (Jensen *et al.*, 2005). Changes in nutritive value (including starch and organic matter digestibility (OMD)) during maturation have been studied extensively (Ettle & Schwarz, 2003; Cone *et al.*, 2008; Arriola *et al.*, 2012). As the maize nutritive value changes during maturation, the optimal harvest date (H_{opt}) is a compromise between whole-crop DM yield, ear DM yield or starch concentration, OMD and a whole-crop DM concentration that is appropriate for storage (Wiersma *et al.*, 1993; Barrière *et al.*, 1997). At the animal level, the goal is to maximize forage intake, digestion and milk/meat production (Bal *et al.*, 1997; Phipps *et al.*, 2000). Several independent studies recommend harvesting maize when the whole-crop DM concentration is between 30 and 35% (Johnson *et al.*, 1999; Phipps *et al.*, 2000; Hetta *et al.*, 2012). Yield, quality and performance indices remain at 95% of their optimum with decreasing moisture concentration until whole-crop DM concentration equals 42% (Darby & Lauer, 2002).

In an ideal variety testing system, each variety would be harvested at a comparable maturity stage. In reality, however, the feasibility of such a strategy is limited due to a number of practical, organizational and economic constraints. A limited number of studies compare varieties at a fixed whole-crop DM concentration (Hetta *et al.*, 2012). Most studies comparing variety performance are done by harvesting all varieties at a single date, which results in a comparison at different DM concentrations (Schwab *et al.*, 2003; Wilkinson & Hill, 2003; Cone *et al.*, 2008). The single harvest date usually corresponds with the date where a reference variety reaches the recommended whole-crop DM concentration. Under this testing system, only a limited proportion of the tested varieties are harvested at their H_{opt}. It is as yet unclear whether a harvest window can be found that would guarantee a stable variety rank. Within such a harvest window, the variety rank based on a single harvest date would equal the variety rank based on harvesting all varieties at their H_{opt}.

We hypothesized that **a single harvest date suffices to compare the nutritive value between varieties when this single harvest date is located within a well-defined harvest window (H3)**. This chapter answers following research questions (**RQ11**) "What is the optimal harvest date, calculated as a compromise between yield, starch concentration and OMD?" and (**RQ12**) "How large is the harvest window, calculated as a set of harvest dates with a variety rank similar to the variety rank at the optimal harvest date?" (Figure 5.1). A dataset of the University College Ghent was used to develop and calculate the optimal harvest date and harvest window. Whole-crop results from the harvest-date trial were used to validate these first results.

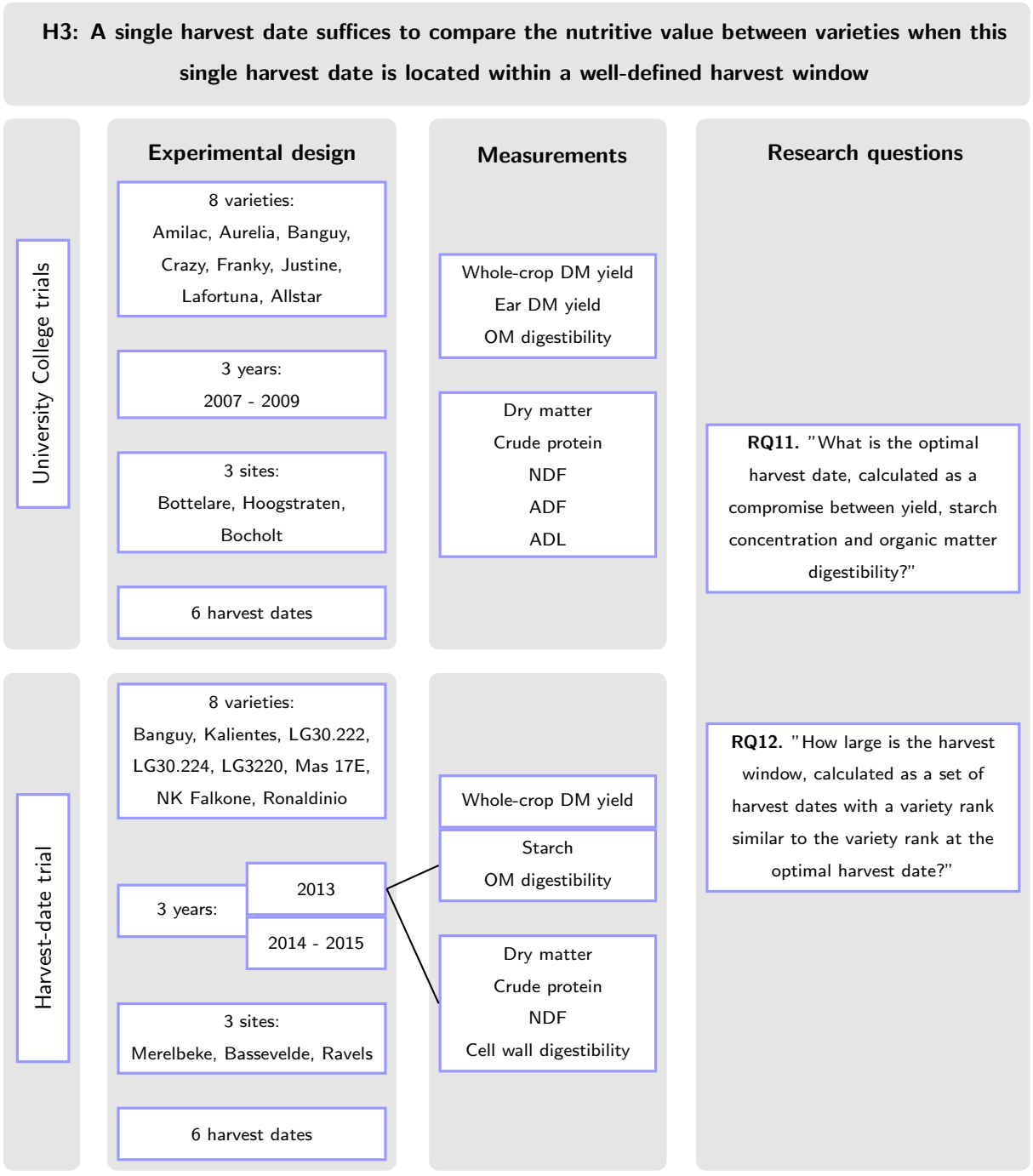


Figure 5.1: Schematic presentation of the research linked to H3. All measurements were applied to all experimental material, except for the relations indicated by a line. When a line is shown, performances were limited to the relations indicated by a line

5.2 Materials and methods

The harvest window was studied using a dataset of the University College Ghent. Our harvest-date trial was used to validate these first results. For the harvest-date trial, the experimental design (including choice of variety, harvest dates, sites and years); sampling method and determination of the maize nutritive value can be found in the chapter "general materials and methods" (Chapter 2). Materials and methods described below only describe the dataset of the University College Ghent.

Experimental site, design and plant material

The dataset of the University College Ghent consisted of eight varieties of silage maize grown on three experimental fields (Bottelare (50°58'N, 3°45'E, 30 m asl), Hoogstraten (51°24'N, 4°46'E, 21 m asl) and Bocholt (51°10'N, 5°34'E, 42 m asl)) in Flanders (the northern part of Belgium) during 3 consecutive years (2007-09). The field experiments were set up in the framework of the 'Flemish Agricultural Centre for fodder crops' programme. The three sites were characterized by different soil types: sandy loam, loamy sand and sand in Bottelare, Hoogstraten and Bocholt, respectively. Eight varieties were chosen, representing the variation between varieties available on the Belgian market. Two maturity types were represented: early varieties (Amilac (KWS), Aurelia (Limagrain), Banguy (Limagrain), and Justina (Pioneer)) and late varieties (KWS), Crazy (Innoseeds), Franky (Scam), and Allstar (Limagrain)). The experimental design was a completely randomized block with three replicates. Plots consisted of four rows 12 m long. Row width was 0.75 m and the plant density was 105 000 plants ha^{-1}. Sowing dates were between 18 April and 19 May (depending on site and year). Manure, fertilizers and herbicides were applied according to recommended agricultural practices in line with current Belgian regulations.

Weather conditions

The 2007 growing season was characterized by normal temperatures and above-average precipitation in July followed by normal precipitation in August, September and October. In 2008, the growing season was characterized by normal temperatures, normal precipitation in July (Bottelare), August (Hoogstraten and Bocholt), September and October; above-average precipitation in July (Hoogstraten and Bocholt) and August (Bottelare). The growing season in 2009 was characterized by high temperature and average rainfall in July and October; below-average rainfall in August and September (Table 5.1).

Table 5.1: Monthly average temperature and rainfall from July to October in Bottelare, Hoogstraten and Bocholt in 2007-2009

		Average temperature (°C)			Historic normals	Rainfall (mm)			Historic normals
		2007	2008	2009	(1981-2010)	2007	2008	2009	(1981-2010)
Bottelare	July	17.7	18.4	18.8	18.3	149.2	60.5	82.1	70.7
Hoogstraten		17.9	18.7	19.6	18.7	192.4	131.6	88.5	81.4
Bocholt		17.5	18.1	19.1	18.5	186.6	155.7	110.0	79.5
Bottelare	August	16.8	18.0	19.4	18.0	41.6	119.3	17.2	72.7
Hoogstraten		17.2	18.3	19.9	18.3	80.1	104.6	41.6	77.3
Bocholt		17.0	17.9	18.9	18.0	120.5	101.6	25.7	75.0
Bottelare	September	14.4	14.2	16.3	15.0	74.5	72.5	22.5	69.7
Hoogstraten		14.8	14.5	16.4	15.3	67.4	51.0	17.4	79.1
Bocholt		13.9	13.4	15.4	14.7	70.8	58.3	35.0	69.1
Bottelare	October	10.7	10.6	11.7	11.4	50.7	83.5	71.4	77.1
Hoogstraten		10.7	10.9	11.6	11.5	43.2	75.2	82.8	81.0
Bocholt		9.8	10.2	10.5	10.8	56.5	83.2	141.4	76.0

Harvests and Ontario Units

Six harvest dates (H_x) were applied during plant maturation. At Hoogstraten, a shorter harvest period was applied: harvest dates 2-6 in 2008 and harvest dates 3-6 in 2009. At Bocholt, harvests were limited to harvest dates 1-5 in 2008 and harvest dates 1-4 in 2009. Harvesting was initiated when the kernels of the earliest hybrid, were at the dent stage (R5) (Ritchie *et al.*, 1997) targeting a whole-crop DM concentration of about 25%. The first harvest date coincided with 2392-2480 Ontario Units (OU) depending on site and year (Table 5.2). Subsequent harvests were taken with intervals of about 100 OU, targeting a whole-crop DM concentration of about 40% at the last harvest date.

Table 5.2: Ontario Units (OU) per harvest date, site and year

Harvest	Bottelare			Hoogstraten		Bocholt		
date	2007	2008	2009	2008	2009	2008	2009	Mean
H_1	2466	2475	2392			2480	2434	2449
H_2	2580	2596	2541	2548		2594	2580	2573
H_3	2688	2683	2677	2662	2603	2742	2713	2681
H_4	2830	2759	2811	2746	2737	2824	2840	2792
H_5	2868	2829	2968	2824	2861	2915		2878
H_6	3045	2933	3059	2896	2971			2981

At each harvest date, all varieties present at the site were harvested on the same day. Fresh yield was measured per plot by weighing all the plants from the two inner rows over a length of 8 m. Plants were cut by hand 10 cm above soil level. Five representative plants were chosen randomly and split into stover and ears (dehusked). Ears were dried at 75 °C for 16 h followed by 5 h at 105 °C to determine ear DM concentration and DM yield. Another five randomly chosen representative plants were chopped to determine DM concentration (72 hours at 65 °C) and quality parameters of whole-crop material. The dry chopped material was milled over a 1-mm screen using a cutting mill (Retsch Model PK 1000).

Determination of the nutritive value

Chemical parameters were measured using near-infrared spectroscopy (NIRs) collected at 1100 to 2500 nm at 4-nm intervals using an Infralyzer 500 spectrophotometer (Bran Luebbe, Norderstedt, Germany) (De Boever *et al.*, 2002). Calibration equations were provided by the Walloon Agricultural Research Centre in Gembloux (Belgium). Samples of the University College Ghent were analysed for crude protein (CP), neutral detergent fibre (NDF), acid detergent fibre (ADF), acid detergent lignin (ADL) and OMD. Determination coefficients between laboratory analyses and NIRs predictions (R^2), as well as standard errors of calibration were, respectively: 0.92 and 3.6 for CP, 0.93 and 17.1 for NDF, 0.95 and 11.5 for ADF, 0.84 and 3.9 for ADL, and 0.92 and 17.0 for OMD. Calibration equations were validated each year with laboratory analyses of Belgian maize samples. The samples in the calibration and validation set were subjected to standard wet chemical analyses. CP concentration was determined by the Kjeldahl method. The determination of NDF was based on the laboratory procedures given by Goering & van Soest (1970) using heat-stable amylase and sodium sulfite. In determining ADF and ADL, the laboratory procedures given by Goering & Van Soest (1970) were followed. The determination of OMD was based on the *in vitro* cellulase technique (De Boever *et al.*, 1997).

5.3 Determination of the harvest window

The harvest window is defined as the set of harvest dates that result in a stable variety rank. The harvest window was calculated according to the methodology presented in Table 5.3.

Table 5.3: Steps to calculate the harvest window

	For each	Calculation	Output
STEP 1	Variety∗ x Year† x Site§	Calculate from the available HDs the optimal HD(s) (H_{opt}) where whole-crop DM yield, ear DM yield (or starch concentration) and OMD were calculated as not significantly different from the date with maximal values, statistically secured by a Tukey test	Optimal HD(s) (H_{opt})
STEP 2	Variety∗ x Year† x Site§ x Parameter#	Determine the mean value of each parameter at H_{opt}	Mean value at H_{opt}
STEP 3	Variety∗ x Year† x Site§ x Parameter# x HD‡ x Replicate	Determine for each HD the difference between the actual value of a parameter and its mean value at H_{opt}	Deviation of the actual fresh value to the mean value at H_{opt}
STEP 4	Parameter#	Perform an ANOVA using differences defined in Step 3 as independent variables with the factors variety (V), harvest date (HD), year (Y) and all interactions. In case of interaction HD x V, HD x V x Y, HD x V x S, HD x V x Y x S, the ANOVA is iteratively calculated by stepwise eliminating HD deviating most from H_{opt}. The calculation is stopped when all interactions including HD x V become non-significant ($P < 0.05$).	Harvest window

∗ Variety = Amilac, Aurelia, Banguy, Justina, Lafortuna, Crazy, Franky, Allstar (Dataset University College Ghent)
= Banguy, Kalientes, LG30.222, LG30.224, LG3220, Mas 17E, NK Falkone, Ronaldinio (Dataset harvest-date trial)

† Year = 2007-2009 (Dataset University College Ghent); 2013-2015 (Dataset harvest-date trial)

§ Site = Bottelare, Hoogstraten, Bocholt (Dataset University College Ghent);
= Merelbeke, Bassevelde, Ravels (Dataset harvest-date trial)

‡ Harvest date = 1,2,3,4,5,6

Parameter= whole-crop DM yield, ear DM yield, NDF, ADF, ADL, OMD (Dataset University College Ghent);
= whole-crop DM yield, starch, CP, OMD, NDF, NDFD (Dataset harvest-date trial)

DM, dry matter; CP, crude protein; OMD, organic matter digestibility; NDFD, cell wall digestibility

5.4 Results

5.4.1 Dataset University College Ghent

Whole-crop dry matter concentration and response to Ontario Units

Whole-crop DM concentration increased linearly during maturation (Table 5.4 and Figure 5.2(a)). At the first date, corresponding with an average of 2449 OU, early varieties showed a DM concentration between 25.6 and 28.1% while late varieties had a DM concentration between 22.6 and 25.2%. The increase in DM concentration was on average 1.8% units per 100 OU with a significant difference between Aurelia (2.2% units per 100 OU) and Lafortuna (1.6% units per 100 OU). The last maturity stage, corresponding with 2981 OU and referred to as harvest date 6, had a DM concentration of 31.0 to 40.6%. The DM concentration difference between all compared varieties at any harvest date increased during maturation: 5.5% at harvest date 1 to 9.6% at harvest date 6. Whole-crop and ear DM yield increased until about 2900 OU (Figure 5.2(b) and (c)). The OMD of early and late varieties increased during the whole grain-filling period (Figure 5.2(d)).

Table 5.4: Regression equations for whole-crop dry matter (DM) concentration, whole-crop DM yield, ear DM yield and organic matter (OM) digestibility. Data were pooled across years (2007, 2008, 2009), sites (Bottelare, Hoogstraten, Bocholt), hybrid (Early= Amilac, Aurealia, Banguy, Justina; Late= Lafortuna, Crazy, Franky, Allstar), and replication (n=84) and regressed against Ontario Units (x) (n=6)

Parameter	Regression equation Early varieties	R^2	Regression equation Late varieties	R^2
Whole-crop DM concentration (%)	$-22.0 + 0.020x$	0.87	$-16.9 + 0.017x$	0.86
Whole-crop DM yield (t ha^{-1})	$602 - 0.7x + 2.8 \times 10^{-4}x^2$ $-3.7 \times 10^{-8}x^3$	0.80	$125 - 0.16x + 7.3 \times 10^{-5}x^2$ $-1.1 \times 10^{-8}x^3$	0.78
Ear DM yield (t ha^{-1})	$533 - 0.6x + 2.5 \times 10^{-4}x^2$ $-3.2 \times 10^{-8}x^3$	0.86	$269 - 0.3x + 1.4 \times 10^{-4}x^2$ $-2.0 \times 10^{-8}x^3$	0.87
OM digestibility (g kg^{-1}OM)	$-17.6 + 0.45x - 7.1 \times 10^{-5}x^2$	0.60	$-47.6 + 0.75x - 1.2 \times 10^{-4}x^2$	0.68

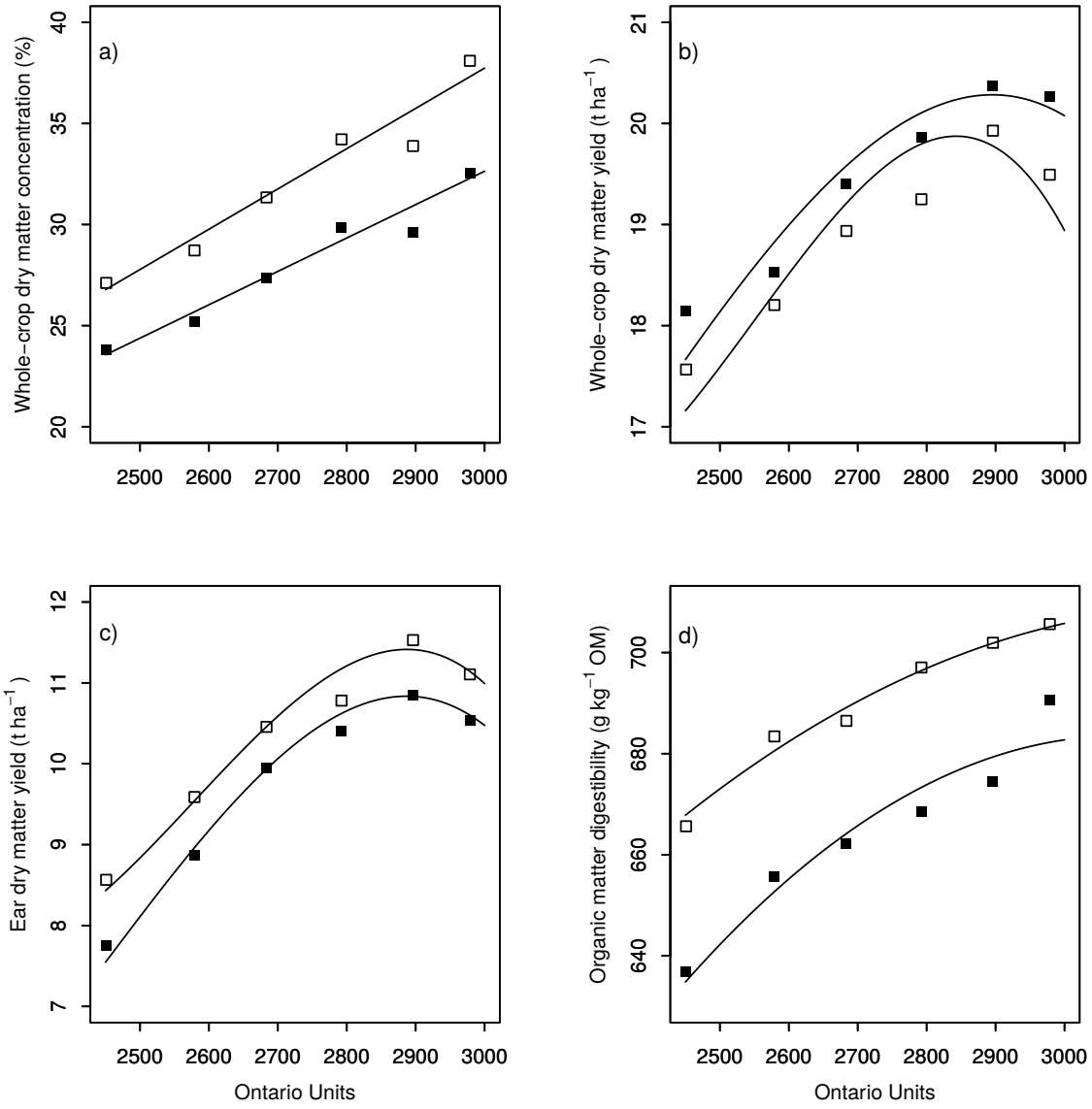


Figure 5.2: Relationship between (a) whole-crop dry matter concentration, (b) whole-crop dry matter yield, (c) ear dry matter yield, and (d) organic matter digestibility with Ontario Units. Data are reported for early varieties (❑) and late varieties (■). Each data point is the mean across four hybrids, three replicates, three years and three sites. Equations and coefficients of determination (R^2) are reported in Table 5.4.

Optimal harvest date (H_{opt})

Optimal harvest dates per variety, site and year are presented in Table 5.5. The whole-crop DM concentration that corresponded with H_{opt} ranged from 26-39% across varieties, years and sites. Within varieties, a range of 6-12% was measured between the years and sites. The mean (S.D.) whole-crop DM concentrations at H_{opt} per variety were 30.7 (2.6) for Crazy; 31.1 (3.1) for Franky; 31.9 (3.1) for Allstar; 33.4 (4.3) for Banguy; 33.6 (3.0) for Lafortuna; 34.0 (4.2) for Justina; 34.7 (3.7) for Aurelia and 35.2 (2.6) for Amilac. Between maturity types, whole-crop DM concentration at H_{opt} was significantly greater for early varieties (34.2%) compared to late varieties (32.0%).

Table 5.5: Optimal harvest dates (H_{opt}) per variety, site and year (presented by grey lanes) with corresponding dry matter (DM) concentration range

Site	Year	Early variety	Harvest date 1 2 3 4 5 6	DM concentration range (%)	Site	Year	Late variety	Harvest date 1 2 3 4 5 6	DM concentration range (%)
Bottelare	2007	Amilac		34.5	Bottelare	2007	Lafortuna		30.4
	2008			32.5 - 32.6		2008			29.9 - 32.0
	2009			34.4 - 39.0		2009			30.0 - 37.6
Hoogstraten	2008			33.5 - 35.4	Hoogstraten	2008			34.3 - 35.3
	2009			32.4		2009			37.4
Bocholt	2008			36.8 - 37.5	Bocholt	2008			34.6 - 35.9
	2009			38.7		2009			30.2 - 37.1
Bottelare	2007	Aurelia		28.1 - 39.0	Bottelare	2007	Crazy		31.9
	2008			28.3 - 36.7		2008			30.2
	2009			33.9 - 37.2		2009			29.5 - 33.5
Hoogstraten	2008			36.6 - 37.1	Hoogstraten	2008			27.3 - 30.8
	2009			34.8		2009			28.9 - 32.7
Bocholt	2008			37.0 - 37.9	Bocholt	2008			30.4 - 30.4
	2009			33.4		2009			29.7 - 34.6
Bottelare	2007	Banguy		26.4 - 34.8	Bottelare	2007	Franky		30.1
	2008			30.3 - 32.8		2008			28.5 - 29.3
	2009			34.3 - 38.3		2009			28.3 - 33.7
Hoogstraten	2008			33.7 - 34.3	Hoogstraten	2008			27.3 - 30.8
	2009			27.9 - 37.7		2009			33.4
Bocholt	2008			36.6 - 38.3	Bocholt	2008			29.0 - 31.0
	2009			35.6 - 37.8		2009			29.7 - 36.7
Bottelare	2007	Justina		26.7 - 35.8	Bottelare	2007	Allstar		33.3
	2008			30.9 - 34.5		2008			26.1 - 31.4
	2009			29.6 - 38.8		2009			
Hoogstraten	2008			33.5 - 34.0	Hoogstraten	2008			28.5 - 33.3
	2009			38.0		2009			29.4 - 34.8
Bocholt	2008			32.7 - 38.0	Bocholt	2008			32.9 - 34.4
	2009			34.4		2009			35.9

Variety ranks at H_{opt} differed per parameter: clear differences among the varieties were detected for all parameters except for CP concentration (Table 5.6). Banguy, Justina and Allstar had the smallest whole-crop DM yield, but these varieties had the best values for OMD.

Table 5.6: Variety rank at the optimal harvest date (H_{opt}) based on Ontario Units (OU), whole-crop dry matter (DM) concentration, whole-crop DM yield, ear DM yield, crude protein (CP), NDF, ADF, ADL and organic matter digestibility (OMD) as means of the sites Bottelare, Hoogstraten, Bocholt and the years 2007, 2008, 2009

Variety	OU	Whole-crop DM concentration (%)	Whole-crop DM yield (t ha^{-1})	Ear DM yield (t ha^{-1})	CP (g kg^{-1}DM)	NDF (g kg^{-1}DM)	ADF (g kg^{-1}DM)	ADL (g kg^{-1}DM)	OMD (g kg^{-1}OM)
Early varieties									
Amilac	2904	35.5	20.0	11.2	70	414	245	31	695
Aurelia	2833	34.7	20.1	11.4	71	414	248	30	695
Banguy	2844	33.4	18.9	10.8	71	402	235	28	716
Justina	2773	33.7	18.9	11.0	70	409	241	30	700
Late varieties									
Lafortuna	2881	33.7	19.8	11.0	71	405	237	28	715
Crazy	2871	30.7	20.0	10.6	72	431	258	32	683
Franky	2876	31.1	19.8	9.8	71	439	263	32	671
Allstar	2831	31.9	19.1	10.4	71	407	240	29	705
S.E.D	57	1.53	0.74	0.46	3.0	19.4	13.8	1.9	20.7

S.E.D, standard error of difference

Harvest window

When all varieties were considered together, the harvest window for the parameters whole-crop DM yield and CP concentration included all harvest dates (Table 5.7). For ear DM yield, NDF, ADF and OMD the harvest window included harvest dates 2-6; for ADL it covered harvest dates 4-6. Therefore, the smallest harvest window that comprised all parameters consisted of harvest dates 4-6. This smallest harvest window corresponded with a whole-crop DM concentration of 28.1-40.6% for all varieties.

Table 5.7: Harvest window for all varieties indicating harvest dates with a stable variety rank

	Harvest date						DM concentration of the
	1	2	3	4	5	6	extreme harvest dates (%)
Whole-crop DM yield	■	■	■	■	■	■	22.6 - 40.6
Ear DM yield		■	■	■	■	■	24.3 - 40.6
Crude protein	■	■	■	■	■	■	22.6 - 40.6
NDF		■	■	■	■	■	24.3 - 40.6
ADF		■	■	■	■	■	24.3 - 40.6
ADL				■	■	■	28.1 - 40.6
OM digestibility		■	■	■	■	■	24.3 - 40.6
All parameters				■	■	■	28.1 - 40.6

The harvest window for early varieties for whole-crop DM yield comprised all harvest dates; for CP concentration and OMD, harvest dates 2-6; for ear DM yield, NDF and ADF, harvest dates 3-6; and for ADL, harvest dates 4-6 (Table 5.8). Consequently, the smallest harvest window for early varieties considering all parameters comprised harvest dates 4-6 and represented a whole-crop DM concentration of 33.2-40.6%. The harvest window for late varieties considering all parameters covered all harvest dates and represented a whole-crop DM concentration of 22.6-34.5%.

Table 5.8: Harvest window for early and late varieties, indicating harvest dates with a stable variety rank

		Harvest date						DM concentration of the
		1	2	3	4	5	6	extreme harvest dates (%)
Early varieties	Whole-crop DM yield							25.6 - 40.6
	Ear DM yield							30.5 - 40.6
	Crude protein							27.6 - 40.6
	NDF							30.5 - 40.6
	ADF							30.5 - 40.6
	ADL							33.2 - 40.6
	OM digestibility							27.6 - 40.6
	All parameters							33.2 - 40.6
Late varieties	Whole-crop DM yield							22.6 - 34.5
	Ear DM yield							22.6 - 34.5
	Crude protein							27.6 - 40.6
	NDF							22.6 - 34.5
	ADF							22.6 - 34.5
	ADL							22.6 - 34.5
	OM digestibility							22.6 - 34.5
	All parameters							22.6 - 34.5

5.4.2 Validation: harvest-date trial

Changes in nutritive value during maturation of the harvest-date trial are described in Chapter 4.2; regression equations are given in Table 4.6 and shown in Figure 4.6.

Optimal harvest date (H_{opt})

Optimal harvest dates per variety, site and year are presented in Table 5.9. The whole-crop DM concentration that corresponded with H_{opt} ranged from 27-40% across varieties, years and sites. Within varieties, a range of 8.5-12% was measured between the years and sites. The mean (S.D.) whole-crop DM concentrations at H_{opt} per variety were 35.5 (2) for Banguy; 35 (2.9) for Kalientes; 34.8 (2.1) for LG30222; 36 (2.8) for LG30224; 33.2 (2.5) for LG3220; 35.4 (2.7) for MAS 17E; 35.6 (2.8) for NK Falkone and 34.5 (2.2) for Ronaldinio.

Variety ranks at H_{opt} differed per parameter: clear differences among the varieties were detected for all parameters (Table 5.10). Banguy had the smallest DM yield, but this variety had the best value for OMD.

Table 5.9: Optimal harvest dates (H_{opt}) per variety, site and year (presented by grey lanes) with corresponding DM concentration range

Site	Year	Variety	Harvest date 1 2 3 4 5 6	DM concentration range (%)	Site	Year	Variety	Harvest date 1 2 3 4 5 6	DM concentration range (%)
Merelbeke	2013	Banguy		31.5 - 39.2	Merelbeke	2013	LG30.222		32.4
	2014			34.0 - 35.7		2014			34.6 - 39.1
	2015			34.5 - 36.1		2015			33.1 - 34.0
Bassevelde	2013			32.6 - 39.2	Bassevelde	2013			31.8 - 36.5
	2014			36.3 - 36.5		2014			28.3 - 33.7
	2015			32.4 - 37.3		2015			33.7 - 37.8
Ravels	2013			29.3 - 35.1	Ravels	2013			29.8 - 39.2
	2014			39.6		2014			34.3 - 38.5
	2015			34.8 - 36.5		2015			36.2
Merelbeke	2013	Kalientes		31.0 - 32.3	Merelbeke	2013	LG3220		30.1 - 39.0
	2014			35.4 - 36.8		2014			35.1 - 37.5
	2015			30.5 - 33.7		2015			33.6 - 35.1
Bassevelde	2013			31.6 - 34.3	Bassevelde	2013			30.3 - 32.1
	2014			28.1 - 36.8		2014			32.7
	2015			33.3 - 39.4		2015			34.9 - 36.5
Ravels	2013			38.0	Ravels	2013			26.9 - 35.3
	2014			38.4		2014			28.2 - 37.6
	2015			32.4 - 38.8		2015			28.4 - 35.8
Merelbeke	2013	LG30224		31.8 - 39.1	Merelbeke	2013	Mas 17E		30.3 - 38.9
	2014			38.4		2014			37.7 - 38.9
	2015			34.0 - 37.3		2015			32.4 - 34.1
Bassevelde	2013			30.6	Bassevelde	2013			32.2 - 33.4
	2014			37.0 - 37.3		2014			34.1 - 36.9
	2015			31.1 - 37.0		2015			32.7 - 36.4
Ravels	2013			31.1 - 38.4	Ravels	2013			29.8 - 35.1
	2014			38.8 - 39.7		2014			40.0
	2015			36.7		2015			37.1 - 37.6
Merelbeke	2013	Ronaldinio		32.0 - 38.8	Merelbeke	2013	NK Falkone		31.2 - 39.8
	2014			32.8 - 37.6		2014			30.6 - 39.3
	2015			29.3 - 33.2		2015			31.9 - 35.2
Bassevelde	2013			36.9 - 39.4	Bassevelde	2013			32.7
	2014			28.2 - 35.8		2014			27.8 - 36.6
	2015			32.9 - 38.4		2015			33.9 - 39.4
Ravels	2013			29.9 - 35.5	Ravels	2013			34.7 - 36.6
	2014			29.9 - 37.3		2014			39.9
	2015			36.3		2015			38.8

Table 5.10: Variety rank at the optimal harvest date (H_{opt}) based on Ontario Units (OU), whole-crop dry matter (DM) concentration, whole-crop DM yield, starch, crude protein (CP), organic matter digestibility (OMD), NDF and cell wall digestibility (NDFD) as means of the sites Merelbeke, Bassevelde, Ravels and the years 2013, 2014, 2015

Variety	OU	DM concentration (%)	DM yield (t ha^{-1})	Starch concentration (g kg^{-1}DM)	CP concentration (g kg^{-1}DM)	OMD (g kg^{-1}OM)	NDF concentration (g kg^{-1}DM)	NDFD (g kg^{-1}NDF)
Banguy	3009	35.5	20.8	371	69	771	379	624
Kalientes	2966	35.0	22.2	360	74	746	382	564
LG30.222	2977	34.8	22.8	352	72	751	403	604
LG30.224	3006	36.0	23.4	352	68	772	383	628
LG3220	2978	33.2	20.8	355	75	759	392	604
MAS 17E	3012	35.4	22.0	349	75	748	395	584
NK Falkone	2988	35.6	22.4	354	71	747	400	585
Ronaldinio	2954	34.5	22.6	358	74	756	392	610
S.E.D.	51.9	1.25	1.34	12.7	2.6	10.5	11.3	13.6

S.E.D, standard error of difference.

Harvest window

The harvest window for the parameters whole-crop DM yield, CP, NDF, OMD and NDF digestibility (NDFD) included all harvest dates (Table 5.11). For starch concentration the harvest window included harvest dates 2-6. Therefore, the smallest harvest window that comprised all parameters consisted of harvest dates 2-6. This smallest harvest window corresponded with a whole-crop DM concentration of 28-39% for all varieties.

Table 5.11: Harvest window for all varieties indicating harvest dates with a stable variety rank

	Harvest date						DM concentration of the
	1	2	3	4	5	6	extreme harvest dates (%)
Whole-crop DM yield							25.0 - 39.3
Starch							27.5 - 39.3
Crude protein							25.0 - 39.3
NDF							25.0 - 39.3
OM digestibility							25.0 - 39.3
Cell wall digestibility							25.0 - 39.3
All parameters							27.5 - 39.3

5.5 Discussion

A limited set of eight varieties in the dataset of the University College is not large enough to generalize conclusions. Therefore, the harvest-date trial was conducted to validate the first results.

The harvest period in the dataset of the University College Ghent covered an average whole-crop DM concentration of 22.6-28.1% at the first harvest date and a DM concentration of 31.0-40.6% at the sixth harvest date, with an average of 1.8% units per 100 OU. Our harvest-date trial (from 2013 to 2015) covered a harvest period from 25% DM concentration at the first harvest date to 38% DM concentration at the sixth harvest date. The DM concentration increased with 2.1% units per 100 OU, which is well in line with current practices. Indeed, the increase in DM concentration of the reference variety measured during the evaluation of the Belgian variety trials ranged from 1.5 to 2.5% units per 100 OU in the period from 2007 to 2009 (data not published). The DM concentration range between varieties was 4-7% in the dataset of the University College Ghent, but only 1-3% in the validation dataset. All varieties were harvested at least on one occasion at a whole-crop DM concentration between 30 and 35% recommended by Johnson *et al.* (1999). Averaged over all varieties in the dataset of the University College Ghent, whole-crop DM yield increased from 17.8 to 20 with 1 t ha^{-1} per 100 OU, which is in accordance with results of Arriola *et al.* (2012) and results of the validation dataset. Ear DM yield increased from 8.2 to 11.2 with 0.4 t ha^{-1} per 100 OU and reached a maximum at 2900 OU. This pattern followed by the ear DM yield was also found in the starch concentration of the validation trials.

Instead of assuming a fixed DM concentration at H_{opt}, the optimal harvest dates were defined by optimizing whole-crop DM yield, ear DM yield (or starch concentration) and OMD within a whole-crop DM concentration range at harvest between 25 and 40%, to account for ensiling and conservation requirements. By simultaneously optimizing the whole-crop DM yield, ear DM yield (or starch concentration) and OMD, H_{opt} is a compromise among quantity and quality. Average whole-crop DM concentrations at H_{opt} per variety were between 31 and 36%, within the recommended range suggested by Johnson *et al.* (1999). However, whole-crop DM concentration at H_{opt} varied between years and sites from 26 to 39%. Despite the variation in whole-crop DM concentration at H_{opt}, the variety rank at H_{opt} was statistically comparable with a variety rank based on a fixed DM concentration of 30-35% for each quality parameter. Monitoring DM concentration was a valuable proxy for monitoring OMD and starch to determine H_{opt}. In the dataset of the University College Ghent, whole-crop DM concentration at H_{opt} was significantly greater for early types compared with late types. This difference could not be confirmed by the literature or the validation dataset. To compare whole-crop DM concentration at H_{opt} between maturity types, sampling should continue until the latest harvest date is no longer optimal. In the dataset of the University College Ghent, the latest harvest date was generally indicated as the optimal harvest date for the late varieties, which indicates that the harvest series were stopped too early. This was not the case for the validation dataset because of the smaller difference in DM concentration between varieties.

Values for starch, CP, NDF, ADF, ADL, OMD and NDFD at H_{opt} were numerically comparable with Cone *et al.* (2008), who studied four maize types in the Netherlands on three harvest dates. The nutritive value of the varieties used in the harvest-data trial are described in Chapter 4. At H_{opt}, Banguy and LG30.224 had the highest values for OMD, with respectively a high and low starch concentration and both the highest values for NDFD. Varieties NK Falkone and Mas 17E had below average values for OMD because of the below average starch concentration, high NDF and low NDFD. Even though Kalientes had a high starch concentration, it had the lowest OMD as a result of a low NDFD.

To our knowledge, this is the first attempt to calculate a harvest window by comparing the variety rank at H_{opt} with the rank at a single harvest date. Such a harvest window is expected to be limited because the effect of harvest date is more pronounced in the early stages of ripening (DM from 29 to 32%) than during prolonged ripening (DM from 32 to 39%), as suggested by Cone *et al.* (2008). The harvest window for all varieties and all parameters included harvest dates 4-6 (OU of 2800 to 3000) in the dataset of the University College Ghent. This was confirmed by the validation dataset where a harvest window was found including harvest dates 2-6 (OU of 2750 to 3150). The span of 200 to 400 OU offers a flexible harvest period of about 14 to 28 days. In contrast with the results of Cone *et al.* (2008), no differences were found in the current study between the variety rank of plants with a whole-crop DM concentration ranging from 28.1 to 35.3% (a difference of 7.2% between all compared varieties at harvest dates 4 and 5) and the variety rank of plants with a DM concentration ranging from 31.0 to 40.6% (a difference of 9.6% at harvest date 6). Furthermore, the variety rank based on a single harvest date equals the variety rank based on harvesting all varieties at H_{opt}. Dividing varieties into an early and late group did not change the harvest window. Based on these results, harvesting varieties with a different maturity type on a single date does not jeopardize a consistent variety rank.

The developed strategy of determining a harvest window guarantees the most relevant comparison of variety performances as long as the whole-crop DM concentration of the latest variety is >28.1% and the whole-crop DM concentration of the earliest variety is <40.6% while their difference is a maximum of 7.2%. The Belgian variety trials, currently based on the whole-crop DM concentration of about 34% of a reference variety with average maturity type, correspond well to the conditions described above.

5.6 Conclusions

The current study allowed H_{opt} to be calculated for single varieties differing in earliness and to define a harvest window resulting in a variety rank that was statistically not different to the rank at H_{opt}.

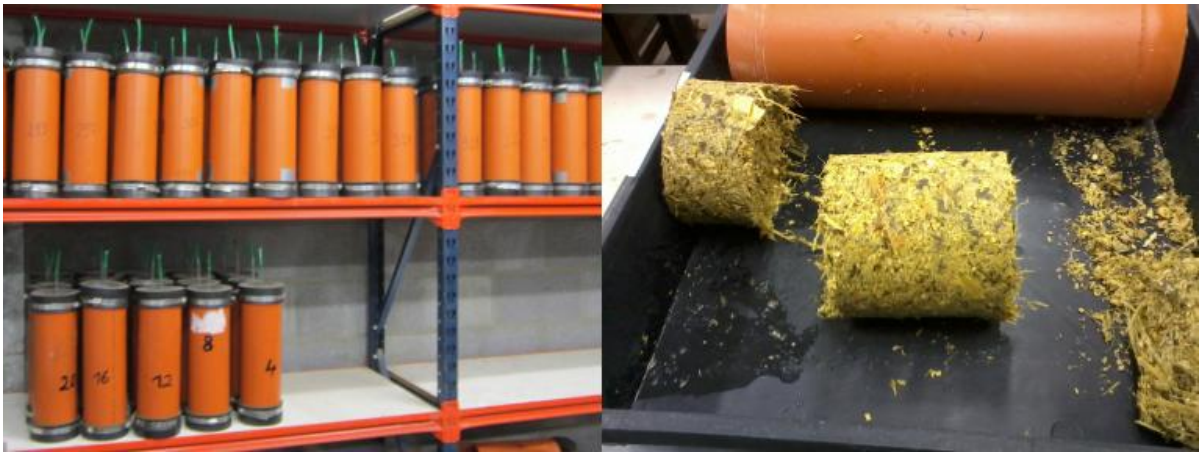
H_{opt}: 31 - 36% DM concentration
Harvest window: 28 - 40% DM concentration

Based on the current results, performing and assessing variety trials with a single harvest date continues to be a scientifically justified practice in Belgium, provided the whole-crop DM concentration is between 28.1 and 40.6% with a maximum difference of 7.2% between all compared varieties at any harvest date.

6

Effect of ensiling on the nutritive value of maize

This chapter is based on: Swanckaert, J., Pannecoucque, J., Van Waes, J., De Boever, J., Haesaert, G., and Reheul, D. Effect of ensiling on variety rank of silage maize. Article submitted with minor revisions to *The Journal of Agricultural Science* (10/11/2016)



6.1 Introduction

Forage maize is nearly exclusively fed as a silage. Ensiling is a common preservation technique based on an anaerobic conversion of water-soluble carbohydrates into organic acids. The stage of maturity of maize at the time of harvest influences the ensiling process and the quality of the maize silage (Filya, 2004; Wambacq *et al.*, 2016). Indeed, as the plant matures, water-soluble carbohydrate levels decrease and starch levels increase as a result of the translocation of sugars from the stover to the ear (Hunt *et al.*, 1989). Therefore at the end of maturation, less fermentable substrate is available for organic acid production and when the forage is harvested too dry, the most digestible part of the crop is used for fermentation (McDonald *et al.*, 1991). At earlier harvest dates, when the dry matter (DM) concentration is below 25%, part of the soluble sugars are lost into effluents.

Although feed analyses are performed on maize silage, reports of official variety trials regarding nutritive value provide data based on analyses of the fresh (non-ensiled) maize. Yet there are a number of publications dealing with the effect of plant maturity on the nutritive value and potential differences between fresh maize and maize silage. Effects of advancing plant maturity on nutritive value have been evaluated for fresh maize (see Chapter 4.2 and Hetta *et al.* (2012)) and maize silage (Ettle & Schwarz, 2003; Cone *et al.*, 2008; Arriola *et al.*, 2012). Maize silage is generally greater in crude protein (CP) and starch concentrations as a percentage of DM than fresh maize because of respiration losses (Cherney *et al.*, 2007). Although not directly fermented by lactic acid bacteria, the fibrous fraction of silages decreases as a result of solubilization of fibre (Der Bedrosian *et al.*, 2012). NDF digestibility (NDFD) of maize silage declines most severely due to ensiling (Darby & Lauer, 2002).

Trials with animals fed with maize silage are carried out to determine the optimal harvest date (H_{opt}), being a compromise between DM intake, digestion and milk production (Bal *et al.*, 1997; Phipps *et al.*, 2000). H_{opt}, as a compromise between quantity and quality, has been studied in fresh maize (see Chapter 4 and Wiersma *et al.* (1993); Barrière *et al.* (1997)) and maize silage (Darby & Lauer, 2002), but to our knowledge no comparisons have been made between fresh maize and maize silage. Differences in nutritive value between fresh maize and maize silage may result in different variety ranks when comparing fresh maize and maize silage. Based on analyses of non-ensiled samples, Swanckaert *et al.* (2016) (see also Chapter 5) demonstrated that changes in forage maize nutritive value during maturation do not jeopardize variety ranks. Darby & Lauer (2002) also reported a stable variety rank during maturation in fresh maize, but the variety rank changed with increasing DM concentrations after ensiling. So the key question remains "to what respect do varieties with a superior fresh nutritive value maintain their characteristics when ensiled?".

This chapter answers three research questions concerning hypothesis **H4: A single harvest date without ensiling simulation suffices to compare maize varieties for their nutritive value.** The research questions were (**RQ13**) "How large is the effect of ensiling on maize nutritive value?", (**RQ14**) "What is the optimal harvest date, calculated from analyses of maize silage?" and (**RQ15**) "How large is the harvest window, calculated as a set of harvest dates for which the variety ranking calculated from analyses of fresh maize corresponds with the variety ranking at the optimal harvest date

calculated from analyses of maize silage?" (Figure 6.1).

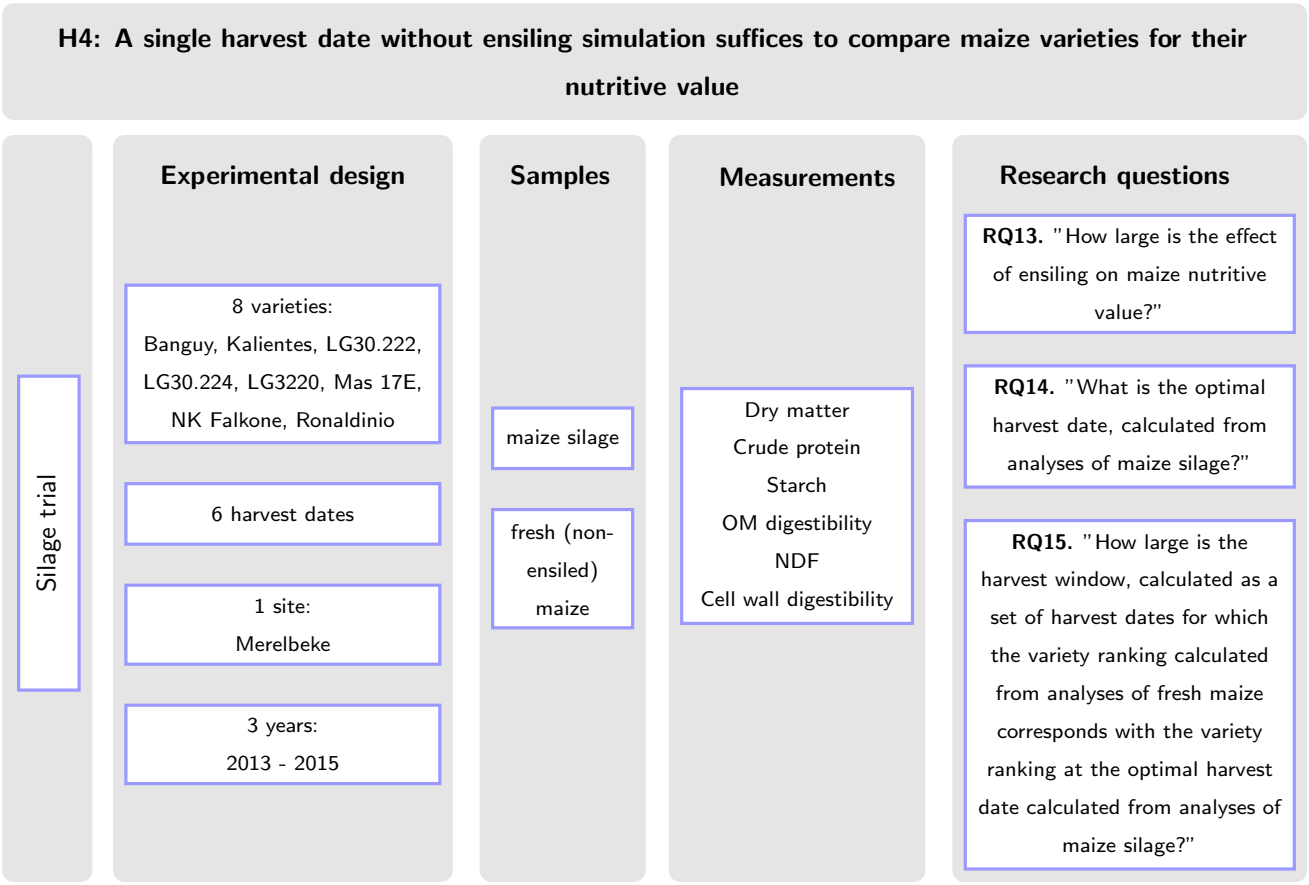


Figure 6.1: Schematic presentation of the research linked to H4

6.2 Materials and methods

The harvest-date trial in Merelbeke was used to study the effect of ensiling on the nutritive value. The experimental design (including choice of variety, harvest date, site and years) is explained in the chapter "general materials and methods" (Chapter 2).

6.2.1 Sampling and ensiling

Ten plants randomly chosen from the inner side of the plot were cut by hand 10 cm above soil level and were completely chopped (6-8 mm). The chopped material was divided into two subsamples. The first was dried at 70 °C for 72 hours to determine DM concentration and chemical parameters on the fresh material. The second was ensiled in airtight laboratory silos of 2.75-L capacity. The silage trial included 432 laboratory silos (8 varieties × 6 harvest dates × 3 years × 3 replicates). Based on estimated DM concentrations at harvest, silos were filled with 200 kg DM m^{-3}. Maize silage was removed from the silos after 20 weeks, frozen and transferred into a freeze drier. Compared to oven drying, freeze drying reduces the loss of volatile organic constituents in fermented forages (Danley & Vetter, 1971). All dried material was milled over a 1-mm screen using a cutting mill (Retsch Model PK 1000).

6.2.2 Determination of nutritive value

Chemical parameters, including CP, starch, neutral detergent fibre (NDF), *in vitro* organic matter digestibility (OMD) and *in vitro* NDFD were estimated using near-infrared spectroscopy (NIRs) collected between 1100 and 2500 nm at 4-nm intervals using a Foss NIRSystems 5000 (Foss, Hillerod, Denmark) and ISIscan (Infrasoft, Port Mathilda, PA, USA) software. Prediction equations were developed for both fresh maize and maize silage samples. Samples used for the NIR analysis were selected to represent the whole spectral and chemical variability in the target population. Statistics relating to NIRs predictions are provided in Table 6.1. The samples in the calibration set were subjected to standard wet chemical analyses, explained in Chapter 2.

Table 6.1: Statistics relating to near-infrared spectroscopy (NIRs) predictions of the fresh maize and maize silage

	Parameter	N∗	Mean	SEC†	SEV(C)‡	R^2
Fresh maize	Crude protein (g kg^{-1}DM)	6529	7.6	0.37	0.38	0.899
	Starch (g kg^{-1}DM)	7283	28.7	1.66	1.68	0.974
	Organic matter digestibility (g kg^{-1}OM)	2902	72.5	1.89	1.93	0.916
	NDF (g kg^{-1}DM)	192	41.7	1.28	1.49	0.924
	Cell wall digestibility (g kg^{-1}NDF)	192	63.3	2.36	2.84	0.816
Maize silage	Crude protein (g kg^{-1}DM)	63	7.4	0.22	0.32	0.844
	Starch (g kg^{-1}DM)	62	35.4	1.71	2.24	0.907
	Organic matter digestibility (g kg^{-1}OM)	63	73.8	1.68	2.01	0.765
	NDF (g kg^{-1}DM)	63	34.0	1.92	2.28	0.675
	Cell wall digestibility (g kg^{-1}NDF)	63	49.1	5.31	7.61	0.785

∗ N, number of data points used to develop NIRs calibration

† SEC, standard error of calibration

‡ SEV(C), standard error of cross validation

The samples in the calibration set were subjected to standard wet chemical analyses. CP concentration was determined by the Kjeldahl method. Starch concentrations were recorded polarimetrically. The determination of OMD was based on the *in vitro* cellulase technique (De Boever *et al.*, 1997). The determination of NDF was based on the laboratory procedures given by (Goering & Van Soest, 1970) using heat-stable amylase and sodium sulfite. NDFD expressed as percentage digestible NDF, was determined after 48h incubation with buffered rumen fluid followed by NDF determination of the undigested residue.

6.2.3 Determination of the harvest window

The harvest window is defined as the set of harvest dates that result in a stable variety rank, adapted from the methodology in Swanckaert *et al.* (2016), described in Chapter 5. The harvest window was calculated according to the methodology presented in Table 6.2.

First, the optimal harvest date or dates (H_{opt}) was/were calculated as the date(s) where starch concentration and OMD were at maximum. H_{opt} was calculated for both fresh maize and maize silage by using a Tukey Test comparing harvest dates with the date showing the greatest values for these two parameters. All dates not significantly different from the date with maximal values were indicated as H_{opt}. Considering the requirements for good preservation, whole-crop DM concentrations were restricted in the range between 25 and 40%. Second, the mean value of each parameter at H_{opt} was calculated, resulting in a variety rank at H_{opt} per parameter. Third, the difference between the mean fresh value of a parameter and its mean silage value at H_{opt} was calculated for each harvest date. Fourth, a harvest window per parameter was calculated based on analyses of variance (ANOVA) using the differences in step 3 as independent variables with the factors variety (V), harvest date (HD), year (Y) and all interactions. Harvest dates were included in the harvest window if interactions HD x V, HD x V x Y were not significant. ANOVAs were iteratively recalculated by stepwise elimination of the harvest date that deviated most from H_{opt} until all interactions including HD x V became non-significant. The remaining dates represented the harvest window.

Table 6.2: Steps to calculate the harvest window (adapted from Swanckaert *et al.* (2016))

	For each	Calculation	Output
STEP 1	Variety∗ x Year†	Calculate from the available HDs the optimal HD(s) (H_{opt}) where starch concentration and OMD were calculated as not significantly different from the date with maximal values for both fresh maize and maize silage, statistically secured by a Tukey test	Optimal HD(s) (H_{opt})
STEP 2	Variety∗ x Year† x Parameter#	Determine the mean silage value of each parameter at H_{opt}	Mean silage value at H_{opt}
STEP 3	Variety∗ x Year† x HD‡ x Replicate x Parameter#	Determine for each harvest date the difference between the actual fresh value of a parameter and its mean silage value at H_{opt}	Deviation of the actual fresh value to the mean silage value at H_{opt}
STEP 4	Parameter#	Perform an ANOVA using differences defined in Step 3 as independent variables with the factors variety (V), harvest date (HD), year (Y) and all interactions. In case of interaction HD x V, HD x V x Y, the ANOVA is iteratively calculated by stepwise eliminating HD deviating most from H_{opt}. The calculation is stopped when all interactions including HD x V become non-significant ($P < 0.05$).	Harvest window

∗ Variety= Banguy, Kalientes, LG30.222, LG30.224, LG3220, Mas 17E, NK Falkone, Ronaldinio

† Year= 2013, 2014, 2015

‡ Harvest date= 1,2,3,4,5,6

Parameter= crude protein, starch, OM digestibility, NDF, cell wall digestibility

6.3 Results

6.3.1 The effect of ensiling on maize nutritive value

Whole-crop DM concentration increased linearly during maturation with 1.9% units per 100 Ontario Units (OU) (Table 6.3 and Figure 6.2(a)). DM concentrations were on average 26% at the first harvest date, corresponding with an average of 2600 OU. The last harvest date resulted in DM concentrations of 36.5 to 39% depending on the variety. The difference in DM concentration between all varieties ranged from 1.6 to 3% units at any harvest date. The DM recovery (expressed as a ratio of kg DM in the silage kg^{-1}DM in at harvest) varied between 953 and 997 g silage DM kg^{-1}DM at harvest. CP concentrations of the fresh maize decreased linearly with later harvest from 80 to 70 g kg^{-1}DM (Table 6.3 and Figure 6.2(b)), while CP concentrations of the maize silage was on average 76 g kg^{-1}DM. Starch concentrations increased quadratically in the fresh maize and maize silage, the difference between fresh maize and maize silage increased from 30 g kg^{-1}DM at 2600 OU to 60 g kg^{-1}DM at 3200 OU (Table 6.3 and Figure 6.2(c)). Average values for OMD were smaller for the maize silage compared to the fresh maize at the first harvest date. Ensiling did not change average values for OMD from harvest date 2 to 6 (Table 6.3 and Figure 6.2(d)). Linear models best explained the relationship between NDF and OU (Table 6.3 and Figure 6.2(e)): NDF concentrations of fresh maize and maize silage decreased with 5.8 g kg^{-1}DM per 100 OU and 8.4 g kg^{-1}DM per 100 OU respectively. NDF concentrations were 54 g kg^{-1}DM greater at 2600 OU for fresh maize compared to maize silage. Different relationships between NDFD and OU were observed for fresh maize and maize silage (Table 6.3 and Figure 6.2(f)). In the fresh forage, NDFD increased linearly with 1.2 g kg^{-1}NDF per 100 OU. In the silage, NDFD decreased following a cubic model. At 3200 OU, the difference in NDFD between fresh maize and silage was 207 g kg^{-1}NDF.

Table 6.3: Regression equations for fresh maize and maize silage nutritive value. Data were pooled across year, variety and replication (n=72) and regressed against Ontario Units (x)(n=6).

Parameter	Regression equation Fresh maize	R^2	Regression equation Maize silage	R^2
Dry matter (%)	$-23.3 + 0.019x$	0.89	$-23.1 + 0.019x$	0.89
Crude protein (g kg^{-1}DM)	$108 - 0.011x$	0.87	$66 + 0.0032x$	0.35
Starch (g kg^{-1}DM)	$-2147 + 1.6x - 2.5 \times 10^{-4}x^2$	0.70	$-2461 + 1.8x - 2.8 \times 10^{-4}x^2$	0.75
OM digestibility (g kg^{-1}OM)	$343 + 0.27x - 6.3 \times 10^{-5}x^2$ $+6.4 \times 10^{-9}x^3$	0.79	$2806 - 2.8x + 1.2 \times 10^{-3}x^2$ $-1.6 \times 10^{-7}x^3$	0.61
NDF (g kg^{-1}DM)	$566 - 0.058x$	0.61	$577 - 0.084x$	0.53
Cell wall digestibility (g kg^{-1}NDF)	$567 + 0.012x$	0.66	$-25570 + 27x - 9.2 \times 10^{-3}x^2$ $+ 1.0 \times 10^{-6}x^3$	0.69

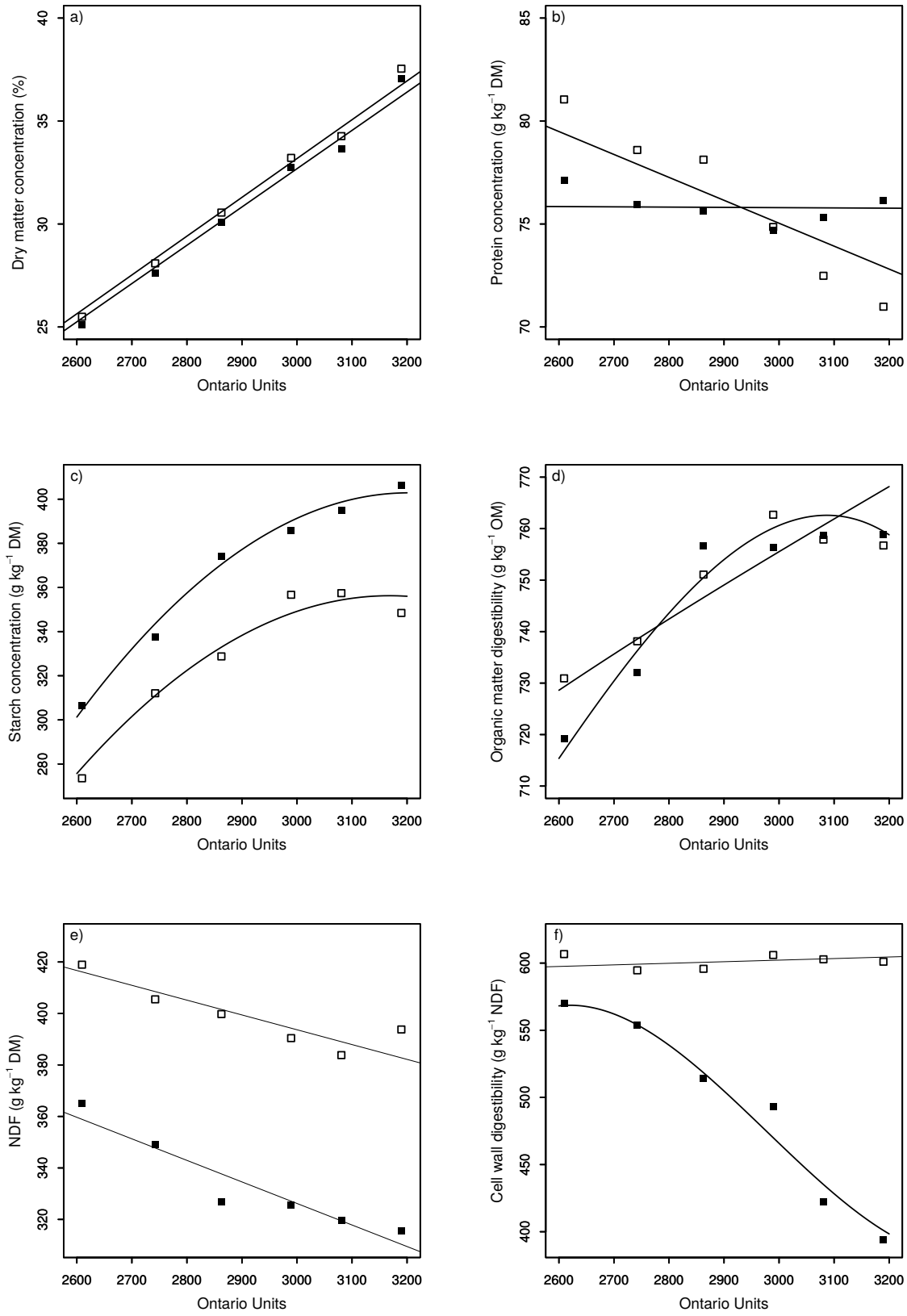


Figure 6.2: Relationship between (a) dry matter concentration, (b) crude protein, (c) starch, (d) organic matter digestibility, (e) NDF and (f) cell wall digestibility with Ontario Units for fresh (❑) and maize silage (■). Each data point is the mean across eight varieties, three replicates and three years. Equations and coefficients of determination (R^2) are reported in Table 6.3.

Ensiling showed a general decreasing trend in S.E. values for CP, starch, OMD and NDF (Table 6.4). However, for NDFD, S.E. values were 2-3 times larger in the maize silage compared to the fresh maize.

Table 6.4: Standard error of the mean (S.E.) for crude protein (CP), starch, organic matter digestibility (OMD), NDF and cell wall digestibility (NDFD) at the each harvest date (indicated by Ontario Units (OU)) for fresh maize and maize silage. Significant levels were calculated by a Levene Test.

OU	CP concentration (g kg^{-1} DM)			Starch concentration (g kg^{-1} DM)			OMD (g kg^{-1} OM)			NDF concentration (g kg^{-1} DM)			NDFD (g kg^{-1} NDF)		
	Fresh	Silage		Fresh	Silage		Fresh	Silage		Fresh	Silage		Fresh	Silage	
2609	0.85	0.59	**	5.31	3.57	**	4.03	2.85	*	3.86	3.87	n.s.	4.53	8.01	***
2742	0.84	0.48	***	3.53	3.22	n.s.	3.65	2.39	***	2.34	2.32	n.s.	4.14	8.21	***
2863	0.95	0.56	***	4.04	3.45	n.s.	3.50	2.73	n.s.	3.37	2.30	**	3.70	5.98	**
2989	0.87	0.52	***	3.03	2.90	n.s.	2.84	2.83	n.s.	2.30	2.23	n.s.	3.21	7.34	***
3081	0.88	0.52	***	3.34	2.90	n.s.	3.33	2.13	**	2.82	2.01	*	3.73	11.17	***
3190	1.04	0.74	**	3.29	4.43	n.s.	3.51	2.90	*	3.29	2.61	n.s.	3.56	7.92	***

6.3.2 The optimal harvest date

Average values for each quality parameter at H_{opt} are shown in Table 6.5 for both fresh maize and maize silage and the corresponding difference. The number of OU to reach H_{opt} depended on variety in the maize silage ($P = 0.002$) but not in the fresh forage ($P = 0.127$). H_{opt} occurred 42 OU earlier to 23 OU later in the maize silage compared to the fresh maize. However, the changes in OU at H_{opt} due to ensiling were not large enough to indicate a significant variety effect (P = 0.077). Accordingly, DM concentrations at harvest differed between varieties when H_{opt} was calculated with data from maize silage ($P = 0.007$), but no variety dependence was found when H_{opt} was calculated with data from fresh maize ($P = 0.264$). At H_{opt}, CP and starch concentrations were always greater in the ensiled product. Changes in protein and starch concentrations varied between 0.2 and 3 g kg^{-1}DM and between 32 and 59 g kg^{-1}DM, respectively, but these changes were not dependent on the variety ($P = 0.341$ and $P = 0.382$). Values for OMD at H_{opt} depended on variety for both fresh maize and maize silage ($P < 0.001$). NDF concentrations at H_{opt} were 62 to 75 g kg^{-1}DM smaller in the maize silage compared to the fresh maize. The decrease in NDFD at H_{opt} varied between 118 and 217 g kg^{-1}NDF. Differences in NDFD between varieties were similar for fresh maize and maize silage, but NDFD in the maize silage was not dependent on variety ($P = 0.175$).

Table 6.5: Evaluation of Ontario Units (OU), dry matter (DM) concentration at harvest, crude protein (CP), starch, organic matter digestibility (OMD), NDF and cell wall digestibility (NDFD) at the optimal harvest date (H_{opt}) for fresh maize, silage maize and corresponding differences as means of the years 2013 - 2015

Variety	OU	DM concentration at harvest (%)	CP concentration (g kg^{-1} DM)	Starch concentration (g kg^{-1} DM)	OMD (g kg^{-1} OM)	NDF concentration (g kg^{-1} DM)	NDFD (g kg^{-1} NDF)
				Fresh maize			
Banguy	3038	34.1	71	364	775	379	628
Kalientes	3031	33.5	75.5	356	751	376	563
LG30.224	2999	32.8	73.3	348	760	398	614
LG30.222	3055	34.5	72	348	774	380	622
LG3220	3076	33.7	75	361	765	386	605
Mas 17E	3070	33.8	77.3	344	749	394	583
Nk Falkone	3025	33.2	72.1	346	744	402	591
Ronaldinio	3027	33.2	74.9	357	760	391	610
P value	0.127	0.264	<0.001	0.157	<0.001	<0.001	<0.001
				Maize silage			
Banguy	3017	34.1	73.6	412	776	305	457
Kalientes	3037	33.2	78.3	397	766	314	445
LG30.224	2957	32.0	74.3	385	758	326	469
LG30.222	3053	34.8	74.2	408	767	305	398
LG3220	3083	33.1	76.8	400	769	311	452
Mas 17E	3042	33.1	78.2	392	759	320	415
Nk Falkone	3012	32.6	74.5	382	747	331	470
Ronaldinio	3018	32.5	75.1	389	750	328	434
P value	0.002	0.007	<0.001	0.012	<0.001	<0.001	0.175
				Silage - Fresh			
Banguy	-21	0.0	2.7	48	1	-73	-171
Kalientes	6	-0.3	2.9	40	15	-62	-118
LG30.224	-42	-0.8	1	37	-2	-73	-145
LG30.222	23	0.4	2.7	59	-7	-75	-217
LG3220	8	-0.6	1.8	39	4	-75	-153
Mas 17E	-28	-0.7	0.9	47	10	-74	-168
Nk Falkone	-13	-0.6	2.4	36	3	-70	-120
Ronaldinio	-9	-0.7	0.2	32	-9	-63	-176
P value	0.077	0.016	0.341	0.382	0.09	0.477	0.035

6.3.3 Harvest window of silage

The harvest window included all harvest dates for the parameters CP concentration, OMD, NDF and NDFD (Table 6.6). For starch concentration, the harvest window covered harvest dates 3 to 6. Consequently, the smallest harvest window comprised harvest dates 3-6 (2832-3117 OU), these harvest dates corresponded with a DM concentration of 29-39%.

Table 6.6: Harvest window (presented by grey lanes) indicating harvest dates with a stable variety rank

	Harvest date						DM concentration of the
	1	2	3	4	5	6	extreme harvest dates (%)
Crude protein							24.4 - 38.9
Starch							29.2 - 38.9
NDF							24.4 - 38.9
OM digestibility							24.4 - 38.9
Cell wall digestibility							24.4 - 38.9
All parameters							29.2 - 38.9

6.4 Discussion

The current trials were conducted with a limited set of eight varieties. These eight varieties differed in earliness (the DM concentration differed with maximum 3% units at any harvest date) and energy source (cell walls or starch), so physiological differences were expected to influence quality parameters, ensiling process and optimum harvest date. Changes in nutritive value due to ensiling were numerically comparable with Lynch *et al.* (2012), who studied six maize varieties in Ireland at three harvest dates. Similar to Johnson *et al.* (2003), we observed a DM recovery value ranging from 953 to 997 g silage DM kg^{-1}DM at harvest depending on variety and harvest date. Effluent losses only occurred at the first harvest date (25-27% DM): they were on average 15 g kg^{-1} maize silage (data not shown). As a result, OMD values for silage were smaller than OMD values for the fresh forage at the first harvest date. If the fermentation occurs without effluent losses, usually a small DM loss associated with respiration of sugars is noticed. Due to this DM loss, the CP concentration in the silage increased at later harvest dates. The values for starch concentrations were generally greater in maize silage compared to fresh forage. This suggests that relatively little breakdown or loss of starch occurred as part of the ensilage process. However, the 20 to 50 g kg^{-1}DM increase in starch concentration may not only be explained by the silage process, but sampling process and determination of the nutritive value may have enlarged these differences. Although the fresh and silage sample originated from the same plants, segregation of chopped particles can lead to a greater/smaller grain fraction in the sample. However, if segregation did occur, it must have been limited because OMD values were similar for fresh maize and maize silage. The determination of the nutritive value was performed with NIRs, using specific calibration equations for fresh maize and for maize silage. We tested a general calibration equation by pooling all samples, but the analytical values did not change.

As hemicellulose is partially hydrolysed under acidic conditions (Filya, 2004), forages with a high cell wall fraction tend to lose more hemicellulose than forages with a small cell wall fraction, leading to a smaller variation in NDF between varieties in the ensiled product. Compared to fresh maize, S.E. of silage was smaller for CP, starch, OMD and NDF. From all quality parameters, ensiling most severely influenced NDFD. The difference in NDFD between fresh maize and maize silage increased with increasing DM concentrations at harvest, in line with Darby & Lauer (2002). S.E. for NDFD of the maize silage was remarkably high. Therefore, we question the reliability of the NIRs-predicted NDFD values for silage. Indeed, statistics of the NIRs predictions showed a high standard error of calibration for NDFD of the maize silage. Therefore, results of NDFD will not be discussed hereafter.

The optimal harvest dates were defined by optimizing starch concentration and OMD, as these parameters are analysed in variety trials in almost all EU countries. Ensiling did change the starch concentration and OMD but the harvest date(s) with maximal values for fresh forage also showed maximal values after ensiling. The average DM concentration for each variety at H_{opt} was between 32 and 35% with an overall mean of 33.4%. This range corresponds with the recommended range suggested by Johnson *et al.* (1999). As the mean OU at H_{opt} were equal for fresh maize and maize silage, the determination of H_{opt} can be performed by frequently measuring starch concentrations and OMD of fresh forage. But one can keep it even more simple: since H_{opt} corresponded with a range in DM concentration at harvest of 32-35%, monitoring DM concentration was a valuable proxy for monitoring OMD and starch to determine H_{opt} in our set of varieties. Ensiling did not change variety ranks at H_{opt}: changes in CP, starch, OMD and NDF due to ensiling were not dependent on the variety. Lynch *et al.* (2012), who calculated differences in nutritive value due to ensiling at three harvest dates, found no effect of variety on differences of starch and OMD, but differences of CP and NDF depended on variety.

A harvest window was defined by Swanckaert *et al.* (2016) as a set of harvest dates where the variety rank at the calculated optimal harvest date is statistically not different from the rank at a given harvest date. By calculating H_{opt} using maize silage, the current study included the effect of ensiling in the harvest window. The harvest window for all parameters included harvest dates 3-6 (OU of 2832-3117). This span of about 300 OU offered a flexible harvest period of about 21 days. The variety rank based on fresh forage at any harvest date within this range equalled the variety rank based on maize silage of all studied varieties at H_{opt}. The Belgian variety trials, currently based on fresh maize at a single harvest date (at a DM concentration of approximately 35% of a reference variety), are corresponding well to the conditions described above. This means that the variety rank currently based on analysing fresh samples is valid; there is no need to ensile the forage and to conduct analyses of maize silage to reliably rank varieties.

6.5 Conclusion

The optimal harvest date of the set of studied forage maize varieties could be predicted by frequently measuring starch concentration and OMD of fresh (i.e. non-preserved) forage. Eventually, harvesting the silage at a DM concentration of 32-35% guaranteed an optimal harvest date. Based on the current results, reporting variety ranks without going through the ensiling process continues to be a scientifically justified practice in the Belgian Official Variety Trials. The key question "to what respect do varieties with a superior fresh quality maintain their characteristics when ensiled?" can be answered as follows: varieties with a superior fresh quality keep their leading position after ensiling, but variety differences become smaller after ensiling; except for NDFD. The varieties with the best nutritive value according to the Official National List Trials continue to be the best when fed as silage to animals.

7

Effect of drought on the nutritive value of maize

7.1 Introduction

Maize yields depend on water availability of the crop, the proportion of water transpired by the crop and water use efficiency of the transpired water (Bänzinger *et al.*, 2000). Plant available water is affected by rainfall (and irrigation), soil surface, soil depth and soil texture. Transpiration is affected by environmental factors such as radiation, temperature, relative humidity and wind; and by plant factors such as stomatal number and size, and leaf area. The water use efficiency, as the ratio between yield and transpiration, is on average 18 kg $ha^{-1}mm^{-1}$ for maize (as a C4 crop) which is significantly higher than for C3 crops such as wheat, rice and cotton (Zwart & Bastiaanssen, 2004). However, with an average rainfall of 450 mm during the growth period of maize in Belgium (from May to September), the average water use efficiency of 18 kg $ha^{-1}mm^{-1}$ would result in a potential yield of 8 t ha^{-1} whereas maize yields of 20 t ha^{-1} and more are achieved in practice. Furthermore, water use efficiency has little to do with drought adaptation because most variation in water use efficiency relate to factors promoting high productivity. There is a positive relation between WUE and potential productivity. But, selecting for high water use efficiency in drought conditions can result in reduced yield (Blum, 1996).

Drought resistance/tolerance can be achieved by a larger investment in roots to improve water absorption and a decrease of leaf area to reduce the area for transpiration (Xu *et al.*, 2010). In addition to drought resistance/tolerance, drought recovery also contributes to the plants ability to cope with drought. The rate and degree of drought recovery depend on previous drought intensity or duration, number of consecutive drying cycles, plant species and variety (Chaves *et al.*, 2003). Maximizing radiation interception and biomass accumulation during the recovery period is achieved by rapid leaf area growth and stimulated by easily available nutritional factors. Accumulation of sugars and amino acids are responsible for repairing the damage caused by water deficiency (Sun *et al.*, 2016). Sugars provide carbon as an immediate energy source in the energy-dependent metabolic pathways. Amino acids, known as recovery-responsive metabolites, are used as a substrate for protein replacement.

Maize is considered more susceptible to water restriction during the reproductive stage than during the vegetative stage. Drought stress at the vegetative stage can drastically reduce vegetation production, but the effect can be compensated for if the maize crop is adequately irrigated during the flowering and grain filling stages (Igbadun *et al.*, 2008). Drought stress during the reproductive stage causes an appreciable delay in silking, while anthesis is not delayed to such an extent. The result is an increase in the anthesis-to-silking interval (Blum, 1996). About 70% of the variation in grain yield is accounted for by variation in anthesis-to-silking interval (Bolaños & Edmeades, 1996). When drought stress is sensed by the plant, its response is to decrease the assimilate partitioning to the developing ear. Therefore, drought stress during the ear formation decreases grain yield by an increased barrenness and reduced number of kernels per plant, while kernel weight was less affected by drought (Bolaños & Edmeades, 1996). The differences in yield of plant fractions due to the availability of water may affect the nutritive value of the maize plant. To our knowledge, no studies have been performed to study the effect of drought on maize nutritive value in Europe. The aim of the present work was to evaluate the effects of irrigation in critical growth stages on the nutritive value of the whole-crop maize and plant parts (leaves, stem and ear).

The hypothesis **H5: Drought influences the nutritive value of maize** was studied by answering the research question (**RQ16**): "How large is the effect of drought on the maize nutritive value during maturation?" (Figure 7.1).

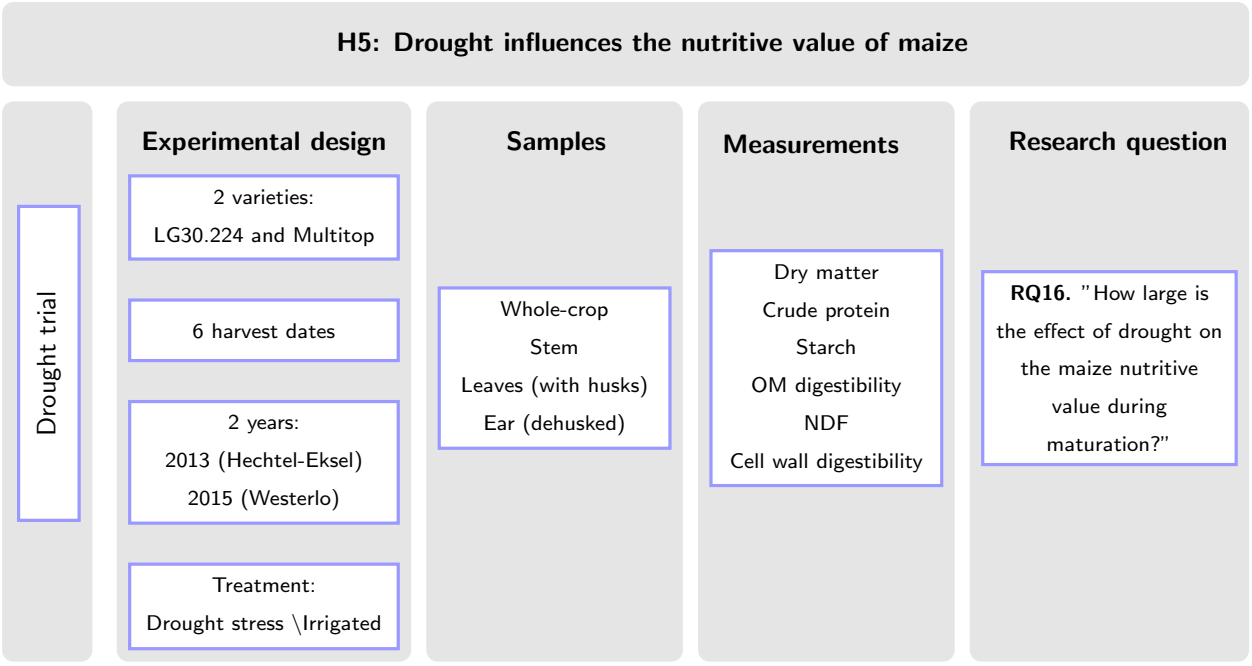


Figure 7.1: Schematic presentation of the research linked to H5

7.2 Materials and methods

Two maize varieties were grown in Hechtel-Eksel in 2013 and Westerlo in 2015 on a sandy soil with 1.6-1.7 % carbon. The two varieties were chosen with a comparable maturation rate: Multitop (Syngenta) and LG30.224 (Limagrain). A field trial of 1 ha was divided into 2 parts, each of which was split in half. Irrigation was allocated to one half of the field. Row width was 0.75 m and the plant density was 105 000 plants ha^{-1}. Sowing dates were 26 April 2013 and 11 May 2015. Manure, fertilizers and herbicides were applied according to recommended agricultural practices in line with current Belgian regulations.

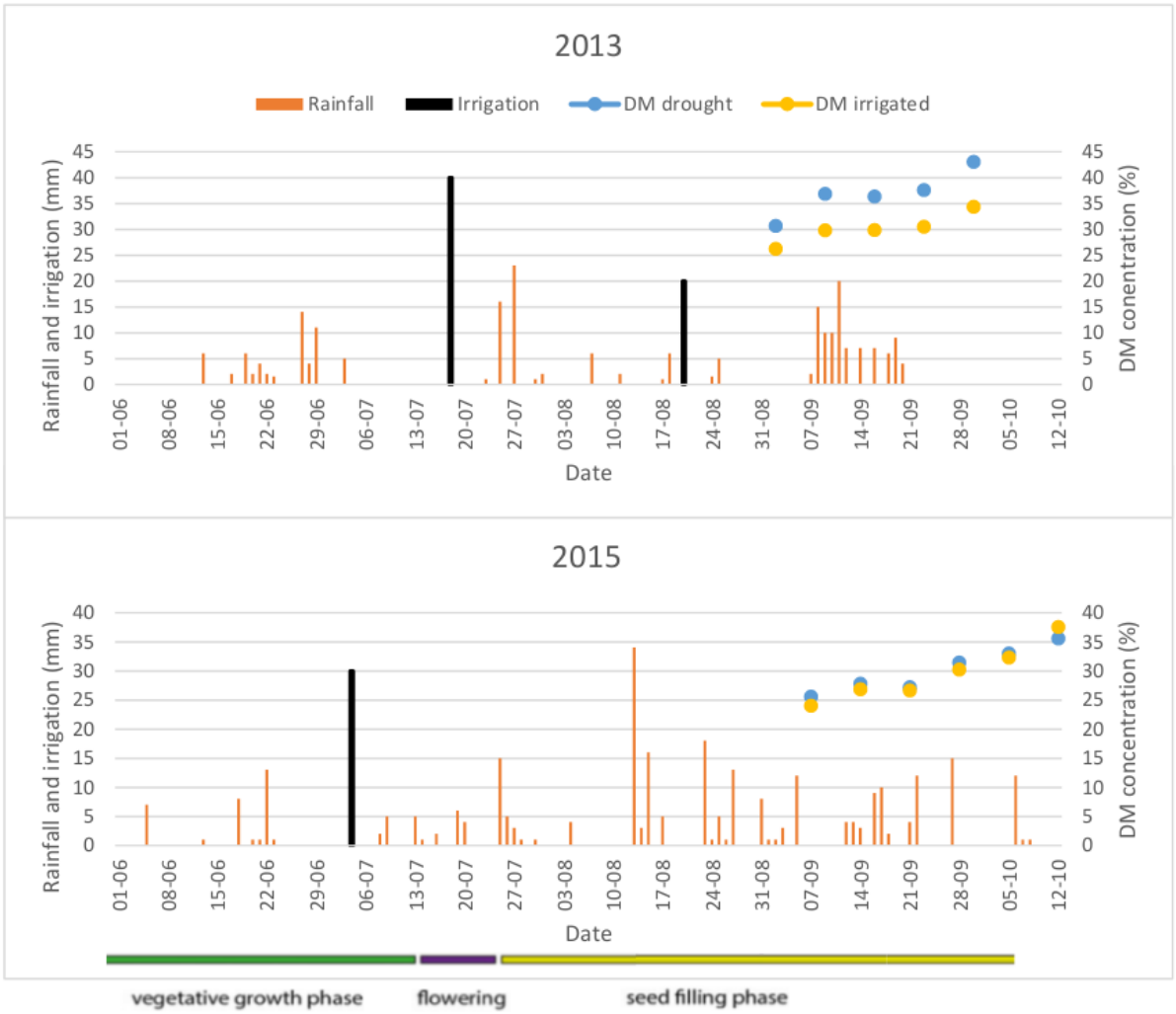
The 2013 growing seasons were characterized by normal daily average temperature in June, a high temperature in July, followed by an average temperature in August and September (Table 7.1). Precipitation was below-average from June to August, followed by average rainfall in September. In 2015, the temperature was average in June, above-average in July and August, and below-average in September. Precipitation was below-average in June and July, above-average in August and average in September.

Table 7.1: Monthly average temperature and rainfall from June to September in 2013 and 2015 in Hechtel-Eksel

	Average temperature (°C)		Historic normals	Rainfall (mm)		Historic normals
	2013	2015	(1981-2010)	2013	2015	(1981-2010)
June	15.7	16.1	16.1	52.5	32	71.8
July	19.6	18.7	18.4	48	48.5	73.5
August	18.0	18.8	17.8	21.5	108	79.0
September	14.3	13.3	14.6	97	80	69.0

Irrigation was applied twice in 2013: 40 mm was on 18 July (during flowering) and 20 mm on 20 August (during seed filling) (Figure 7.2). Irrigation was applied once in 2015: 30 mm 4 July (during the vegetative stage).

Figure 7.2: Rainfall and irrigation from June to September in 2013 and 2015. Average dry matter (DM) concentrations are shown for the plants with drought stress and the irrigated plants at each harvest date.



Five harvest dates (H_x) were applied during plant maturation in 2013 and six harvest dates were applied in 2015 (Table 7.2). Harvesting was initiated when the kernels of the earliest hybrid, were at the dent stage (R5) (Ritchie *et al.*, 1997) targeting a whole-crop dry matter (DM) concentration of about 25%. The first harvest data coincided with 2386 Ontario Units (OU). Subsequent harvests were taken with intervals of 55-140 OU, targeting a whole-crop DM concentration of about 40% at the last harvest date.

Table 7.2: Ontario Units (OU) per harvest date, site and year

Harvest date	2013	2015	Mean
H_1	2382	2389	2386
H_2	2535	2509	2522
H_3	2639	2620	2629
H_4	2727	2713	2720
H_5	2823	2795	2809
H_6		2864	2864

At each of the harvest dates, whole-crop and plant parts (leaves (with husks), stem and ears (dehusked)) were taken to determine DM concentration, crude protein (CP) concentration, starch concentration, neutral detergent fibre (NDF), organic matter digestibility (OMD) and NDF digestibility (NDFD). Sampling methods and determination of the maize nutritive value are described in the chapter "general materials and methods" (Chapter 2).

7.3 Results

Whole-crop DM concentrations did not differ between varieties in 2013, while a difference of 1.5% was measured between the varieties in 2015 independent of the treatment. The effect of irrigation was only found in 2013: irrigation slowed down maturation (the interaction term HD x Treatment was significant) and mean DM concentrations were smaller with the irrigation treatment (Table 7.3).

Table 7.3: Mean dry matter concentration (%) and effects of: harvest date (HD), variety (Var) and irrigation (Treatment) of the whole-crop, leaves, stem and ear in 2013 and 2015.

	Whole-crop		Leaves		Stem		Ear	
	2013	2015	2013	2015	2013	2015	2013	2015
Drought stress								
LG30.224	36.5	30.5	41.6	26	23.4	18	53.3	53.7
Multitop	35.7	28.8	50.5	22.3	26.7	17.6	50.8	52.1
Irrigated								
LG30.224	30.8	30.3	30.4	26.2	22.2	17.8	50.1	53.9
Multitop	29.5	28.9	27.8	23	21.3	16.8	46	52.6
S.E.	0.61	0.55	1.69	0.64	0.41	0.24	0.8	0.47
Effect (P value)								
HD	< 0.001	< 0.001	< 0.001	< 0.001	0.206	< 0.001	< 0.001	< 0.001
Var	0.407	< 0.001	0.053	< 0.001	0.045	0.041	0.001	0.102
Treatment	< 0.001	0.04	< 0.001	0.297	< 0.001	0.13	< 0.001	0.116
HD x Var	0.599	-	0.004	< 0.001	0.002	0.13	-	-
HD x Treatment	0.04	-	< 0.001	0.003	0.233	0.004	-	0.06
Var x Treatment	0.051	-	0.001	-	0.001	-	-	-
HD x Var x Treatment	0.143	-	0.011	-	0.119	-	-	-

- The parameter was excluded from the statistical model by stepwise simplification

The effect of treatment on CP was significant in each year for each plant part, although different results were found for both years (Tabel 7.4). In 2013, irrigation increased CP of the whole-crop and leaves. In 2015, CP decreased due to irrigation. Generally, treatment differences were larger for Multitop compared to LG30.224.

Multitop had a greater starch concentration in the whole-crop for both treatments compared to LG30.224 (Table 7.5). Effects of irrigation were dependent on the year: irrigation resulted in a smaller or greater starch concentration, in 2013 and 2015 respectively.

Table 7.4: Mean crude protein (g kg^{-1}DM) and effects of: harvest date (HD), variety (Var) and irrigation (Treatment) of the whole-crop, leaves, stem and ear in 2013 and 2015.

	Whole-crop		Leaves		Stem		Ear	
	2013	2015	2013	2015	2013	2015	2013	2015
Drought stress								
LG30.224	49.8	71.9	52.8	74.2	18.4	22.5	63.6	84
Multitop	52.5	79.2	44.6	81.5	19.8	27.7	69.7	95.1
Irrigated								
LG30.224	52.4	65.3	68.6	74	17.2	21.4	59.9	72.6
Multitop	55.1	69.1	74.8	73.8	17.5	22.7	58.2	79.7
S.E.	0.7	0.8	2.56	1.06	0.41	0.47	0.94	1.31
Effect (P value)								
HD	0.009	< 0.001	< 0.001	< 0.001	0.073	0.042	0.079	-
Var	0.032	< 0.001	0.766	0.046	-	< 0.001	0.13	< 0.001
Treatment	0.04	< 0.001	< 0.001	0.02	0.025	< 0.001	< 0.001	< 0.001
HD x Var	-	0.039	-	-	-	0.05	-	-
HD x Treatment	-	0.11	-	-	0.015	-	0.129	-
Var x Treatment	-	< 0.001	0.042	0.03	-	0.012	0.01	-
HD x Var x Treatment	-	-	-	-	-	-	-	-

- The parameter was excluded from the statistical model by stepwise simplification

Table 7.5: Mean starch (g kg^{-1}DM) and effects of: harvest date (HD), variety (Var) and irrigation (Treatment) of the whole-crop and ear in 2013 and 2015.

	Whole-crop		Ear	
	2013	2015	2013	2015
Drought stress				
LG30.224	277	274	527	584
Multitop	308	293	557	599
Irrigated				
LG30.224	241	281	495	587
Multitop	267	317	496	603
S.E.	8.1	6.3	7.6	4
Effect (P value)				
HD	< 0.001	< 0.001	< 0.001	< 0.001
Var	< 0.001	0.003	0.061	0.001
Treatment	< 0.001	0.038	< 0.001	-
HD x Var	-	-	-	0.018
HD x Treatment	-	-	-	-
Var x Treatment	-	-	0.085	-
HD x Var x Treatment	-	-	-	-

- The parameter was excluded from the statistical model by stepwise simplification

Whole-crop and stover OMD were generally greater in LG30.224 compared to Multitop (Table 7.6). No effect of treatment was found on whole-crop OMD in 2013, while stover and ear OMD increased due to irrigation. In 2015, irrigation decreased OMD of the whole-crop and stover.

Table 7.6: Mean organic matter digestibility (g kg^{-1}OM) and effects of: harvest date (HD), variety (Var) and irrigation (Treatment) of the whole-crop, leaves, stem and ear in 2013 and 2015.

	Whole-crop		Leaves		Stem		Ear	
	2013	2015	2013	2015	2013	2015	2013	2015
Drought stress								
LG30.224	735	758	594	669	469	484	886	920
Multitop	705	739	564	652	364	454	889	916
Irrigated								
LG30.224	735	734	612	658	493	423	878	919
Multitop	713	719	617	638	417	352	884	918
S.E.	2.9	3	4.7	4.8	11.5	8.1	1.5	0.9
Effect (P value)								
HD	-	< 0.001	< 0.001	< 0.001	< 0.001	-	< 0.001	< 0.001
Var	< 0.001	< 0.001	0.057	0.001	< 0.001	< 0.001	0.044	0.121
Treatment	-	< 0.001	< 0.001	0.015	0.003	< 0.001	0.005	-
HD x Var	-	-	0.046	0.021	-	-	0.053	0.034
HD x Treatment	-	-	-	0.077	-	-	0.015	-
Var x Treatment	-	< 0.001	0.007	0.828	-	0.085	0.46	-
HD x Var x Treatment	-	-	-	0.125	-	-	0.122	-

- The parameter was excluded from the statistical model by stepwise simplification

In 2013, the effect of irrigation treatment decreased NDF of whole-crop and stover (Table 7.7). In 2015, NDF increased due to irrigation.

Table 7.7: Mean NDF (g kg^{-1}DM) and effects of: harvest date (HD), variety (Var) and irrigation (Treatment) of the whole-crop, leaves, stem and ear in 2013 and 2015.

	Whole-crop		Leaves		Stem		Ear	
	2013	2015	2013	2015	2013	2015	2013	2015
Drought stress								
LG30.224	473	399	761	694	699	652	372	198
Multitop	512	387	794	701	811	675	449	266
Irrigated								
LG30.224	461	415	735	717	668	702	396	214
Multitop	475	409	722	725	743	767	436	286
S.E.	3.9	3.3	8	6.3	11.9	8.2	5.8	5.2
Effect (P value)								
HD	0.017	< 0.001	< 0.001	< 0.001	< 0.001	0.115	< 0.001	0.156
Var	< 0.001	< 0.001	0.277	0.372	< 0.001	0.001	< 0.001	< 0.001
Treatment	< 0.001	0.053	< 0.001	0.005	< 0.001	< 0.001	0.387	0.002
HD x Var	-	-	0.024	0.077	-	-	0.035	-
HD x Treatment	0.132	-	0.007	0.031	0.126	-	0.026	-
Var x Treatment	0.036	0.003	0.015	0.948	0.144	0.112	0.001	-
HD x Var x Treatment	-	-	-	0.076	-	-	0.067	-

- The parameter was excluded from the statistical model by stepwise simplification

The variety LG30.224 had a greater NDFD compared to Mulitop in 2015; no variety differences were found in 2013 (Table 7.8). In 2013, whole-crop and stem NDFD did not differ between irrigation treatments; leaves and ear NDFD increased due to irrigation. In 2015, whole-crop and stem NDFD decreased due to irrigation.

Table 7.8: Mean cell wall digestibility (g kg^{-1}NDF) and effects of: harvest date (HD), variety (Var) and irrigation (Treatment) of the whole-crop, leaves, stem and ear in 2013 and 2015.

	Whole-crop		Leaves		Stem		Ear	
	2013	2015	2013	2015	2013	2015	2013	2015
Drought stress								
LG30.224	698	657	750	806	655	626	819	774
Multitop	691	615	737	795	639	607	818	784
Irrigated								
LG30.224	709	638	764	806	651	599	834	783
Multitop	696	611	760	784	633	581	833	794
S.E.	4.6	4	2.7	2.2	4.5	2.5	2.3	2.7
Effect (P value)								
HD	0.018	< 0.001	-	< 0.001	-	0.017	< 0.001	< 0.001
Var	-	< 0.001	0.096	< 0.001	0.057	< 0.001	-	0.001
Treatment	-	< 0.001	< 0.001	0.066	-	< 0.001	< 0.001	0.002
HD x Var	-	0.015	-	0.037	-	0.475	-	-
HD x Treatment	-	0.174	-	-	-	0.795	-	-
Var x Treatment	-	0.034	-	0.081	-	0.832	-	-
HD x Var x Treatment	-	-	-	-	-	0.134	-	-

- The parameter was excluded from the statistical model by stepwise simplification

7.4 Discussion

The main objective of our study was to investigate the effect of irrigation at a crucial growth stage on the nutritive value of two maize varieties. In both growing seasons, drought stress occurred during the flowering period, in which maize is generally considered most susceptible (Igbadun *et al.*, 2008). In contrast with 2015, the drought period continued during grain filling in 2013. Therefore, results from 2013 related to the combined effect of drought during flowering and grain-filling. Results from 2015 were limited to the effect of drought during flowering, so plants could recover from drought during grain-filling. The timing of drought is expected to have an effect on DM yield. Unfortunately, whole-crop DM yield was not recorded. We recorded ear DM yield in 2015: drought during flowering resulted in a 10% decrease in ear DM yield.

Drought stress induces accumulation of sugars in the stover (Sun *et al.*, 2016). As a consequence, translocation of nutrients from the stover to the ear was limited and DM accumulation halted in the stressed kernels. Islam *et al.* (2012) reported an increase in the harvest index (i.e. proportion of grain in the harvested biomass) with increasing water availability. However, Di Marco *et al.* (2007) reported a similar harvest index between the rain fed and irrigated crop. The results in 2015 indicated an increase in the harvest index: ear DM yield decreased with 10% while plant height decreased with 25% (data not shown). Indeed, starch concentrations increased owing to irrigation in 2015. However, irrigation negatively influenced starch concentrations in 2013. Contrasting results between the two tested years on nutritive value were found, probably related to the water deficiency level and environmental changes during drought period. In 2013, irrigation improved OMD while starch concentrations and NDF decreased and NDFD did not change. Results of 2015 were in agreement with Islam *et al.* (2012) who studied the effect of four irrigation levels (0, 153, 305 and 480 mm of total water) in maize in Australia.

Despite increased starch concentrations, the increase in irrigation level increased NDF and decreased OMD. The apparent discrepancy (i.e., increase in NDF despite an increase in starch concentrations) can be explained by the fast translocation of sugars from the stover to the ear under optimum water availability. Irrigated maize had smaller OMD values compared to drought-stressed maize because NDF increased and NDFD decreased. However, Masoero *et al.* (2013), studying the effect of a water-saving irrigation on nutritive value and digestibility, observed similar OMD, NDF and NDFD values between the irrigation levels.

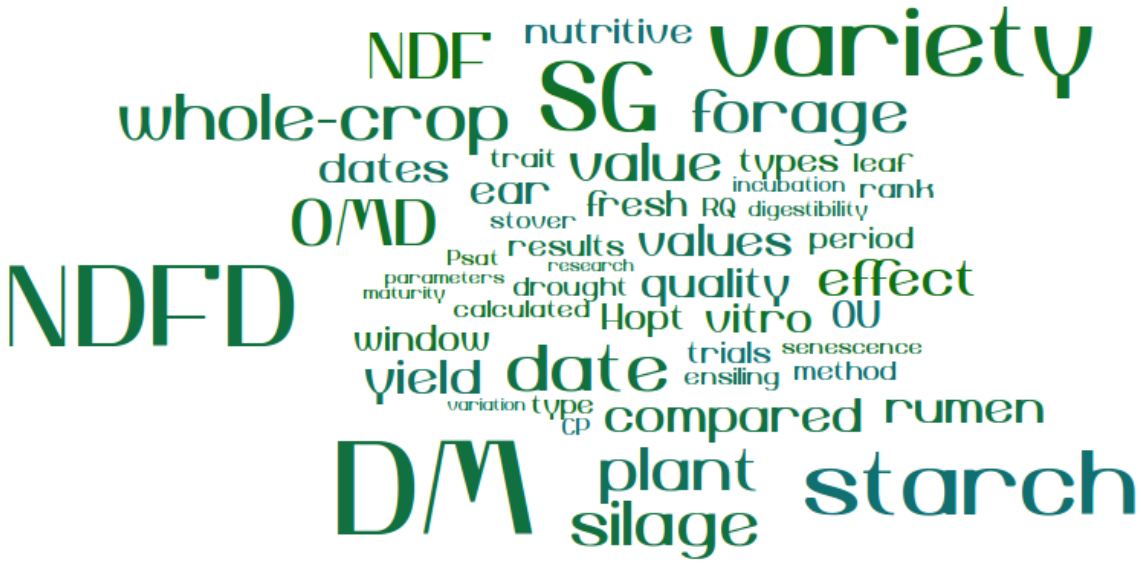
Drought stress during grain-filling might cause induction of premature leaf senescence and might lead to early maturation (Gregerson *et al.*, 2013). Indeed, whole-crop DM concentrations were 6% greater under drought stress in 2013, when the drought period lasted until 2500 OU (harvest date 2). Drought tolerance involves a delayed senescence of the plant, but it is still not clear whether this delay in senescence is causing the drought tolerance or drought tolerance is a consequence of delayed senescence. The stay-green (SG) term, used for varieties showing delayed senescence in the field (See Chapter 2), is associated with maintenance of photosynthetic capacity during the grain-filling period. Even though delayed senescence in SG varieties was observed under non-stressed conditions, it may be associated with drought tolerance (Gregerson *et al.*, 2013).

7.5 Conclusion

Results on the effect of irrigation on maize nutritive value were contradictory between experimental years although the same experimental design was used. The drought period in 2013 included the flowering and grain-filling period, while drought in 2015 was limited to flowering. A drought-stressed maize plant adapts its stover-ear relation. The confrontation with a drought period does not automatically mean that maize nutritive value is negatively influenced.

8

General conclusions



8.1 Feedback to hypotheses and research questions

8.1.1 Hypothesis H1a: Two maize silage varietes (greater NDFD vs more starch) and third treatment with maize meal to bridge the gap in OMD result in similar performances of dairy cattle

RQ1: "How large is the effect of maize energy source on milk production?"
Milk production was not affected by type of maize silage, provided that organic matter digestibility (OMD) is similar and on the condition that the ration is formulated according to the energy requirements and providing enough physical structure to avoid negative effects on rumen fermentation.

RQ2: "How large is the effect of maize energy source on rumen metabolism, nutrient digestibility and methane emission?"
The type of maize silage did not affect pH nor volatile fatty acid composition. Total tract digestibility of the nutrients and N-efficiency were similar between the treatments. The high starch variety had a smaller methane production per day than the high NDF digestibility (NDFD) variety.

RQ3: "Can *in vitro* incubation with rumen fluid be used as an alternative for *in situ* nylon bag technique to rank maize silages according to NDFD?"
In situ rumen NDFD was more than 10% units smaller than *in vitro* NDFD but the difference between the maize types was similar with both methods.

Conclusion: *H1a is supported.*
Our results support the view that one can breed maize varieties of high nutritive value by different ways, either by a high starch concentration or by improving NDFD.

8.1.2 Hypothesis H1b: Measurements of NDFD are suitable for routine evaluation of the nutritive value

RQ4: "What is the best *in vitro* method to determine NDFD?"
The standard *in vitro* incubation with rumen fluid for 48 h continues to be the best practice for *in vitro* NDFD determination. A weak correlation was found between *in situ* NDFD and enzymatic *in vitro* NDFD. The Daisy^{II} technique resulted in poor precision terms.

RQ5: "Can NDFD be estimated based on NDF/starch and OMD?"
A good relationship was found between starch, NDF and OMD. The correlation between NDF and NDFD was close to zero. NDFD can be computed assuming that the non-NDF part of plant material is completely digestible. A more accurate estimation of NDFD was found assuming that only starch is completely digestible.

Conclusion: *H1b is supported.*

The standard *in vitro* determination of NDFD with rumen fluid is time and labour consuming. Alternative methods (enzymatic or Daisy^{II} technique) can save labour but are less precise. Ultimately, calculating NDFD based on starch concentration and OMD suffices to accurately predict NDFD.

8.1.3 Hypothesis H2a: Functional SG plant types can be identified by studying photosynthesis and leaf characteristics

RQ6: "Is the variation in P_{sat}, leaf N concentration, chlorophyll concentration, SPAD and greenness score between varieties large enough to define plant types?"

Yes, the stay-green (SG) type had photosynthetic capacity (P_{sat}) values that were 1 $\mu mol\ m^{-2}s^{-1}$ greater than corresponding values in the normal type, during the whole grain-filling period. Although this difference was significant and found in both experimental years, it only corresponded with a delayed senescence of 2 to 3 days. The proxies for P_{sat} were more discriminating than P_{sat} measurements. A greater chlorophyll concentration and a greater leaf N concentration were responsible for the greater P_{sat} values. Hence, the SG characterization of the studied eight varieties could be performed by studying leaf characteristics.

RQ7: "How large is the effect of the SG trait on photosynthates (sucrose, fructose and starch concentration) in the leaves?"

Leaf sucrose and leaf fructose concentration did not differ between plant types. Leaf starch concentration was 0.07 g $100g^{-1}$ FW greater in the normal varieties compared to SG varieties. Starch accumulation in the leaves in normal varieties inhibited photosynthesis by a feedback system, evidenced by a 0.015 mol $m^{-2}s$ smaller stomatal conductance compared to normal varieties.

RQ8: "How large is the effect of the SG trait on N dynamics and DM yield?"

The SG trait influenced N dynamics during grain filling. Compared to normal varieties, SG varieties incorporated more N into the vegetative tissues and translocated less N from the leaves into the ears. As a result of this lower N translocation, the ears of SG varieties contained 10% less N than the ears of normal varieties, which corresponded with 20 $kg\ ha^{-1}$. This resulted in a 10% smaller ear DM yield in the SG varieties compared to the normal varieties. Whole-crop DM yield was similar between the plant types, but SG varieties retained a larger proportion of the total biomass in the stover compared to normal varieties.

Conclusion: *H2a is supported.*

Two plant types were identified based on photosynthesis. The SG characterization of the studied eight varieties could be performed by studying leaf characteristics. N dynamics were influenced by the SG trait: N concentration shifted between vegetative and generative tissues.

8.1.4 Hypothesis H2b: Variation in maize nutritive value is mainly determined by maturation and SG trait

RQ9: "How does the nutritive value change during maturation?"

During maturation, dry matter (DM) concentration increased linearly with 2% units per 100 Ontario Units (OU). Maize is dependent on N uptake by the roots during grain filling: an extra 30 $kg\ ha^{-1}$ is exported by the whole-crop during the studied period. A maximum starch concentration, reached at 3000 OU coincided with maximum OMD values. The stem and leaves had similar neutral detergent fibre (NDF) values, but NDFD of the stem was 25% smaller than NDFD of the leaves.

RQ10: "How large is the effect of SG trait on maize nutritive value?"

The SG trait had a positive effect on maize nutritive value. SG varieties had a greater starch concentration, greater OMD, smaller NDF and greater NDFD in the whole-crop and stover compared to normal varieties. Nutritive value differences between plant types were most pronounced in the stem.

Conclusion: *H2b is supported.*

Increasing DM concentrations during grain filling affected the maize nutritive value. Despite the greater stover fraction (see RQ8), the SG varieties were more digestible compared to normal varieties.

8.1.5 Hypothesis H3: A single harvest date suffices to compare the nutritive values between varieties when this single harvest date is located within a well-defined harvest window

RQ11: "What is the optimal harvest date, calculated as a compromise between yield, starch concentration and OMD?"

The optimal harvest date (H_{opt}) was defined by optimizing whole-crop DM yield, ear DM yield (or starch concentration) and OMD. Therefore, H_{opt} is a compromise among quantity and quality. Average whole-crop DM concentrations at H_{opt} per variety ranged between 31 and 36%. Monitoring DM concentration is a valuable proxy for monitoring yield, starch and OMD to determine H_{opt}.

RQ12: "How large is the harvest window, calculated as a set of harvest dates with a variety rank similar to the variety rank at the optimal harvest date?"

The harvest window ranged from 2800 to 3100 OU, corresponding with a flexible harvest period of about 21 days. The developed strategy of determining a harvest window guarantees the most relevant comparison of variety performances as long as the whole-crop DM concentration of the latest variety was >28.1% and the whole-crop DM concentration of the earliest variety was < 40.6% while their difference was a maximum of 7.2%.

Conclusion: *H3 is supported.*

Performing and assessing variety trials with a single harvest date continues to be a scientifically justified practice in Belgium.

8.1.6 Hypothesis H4: A single harvest date suffices to compare the nutritive value between varieties without going through the ensiling process

RQ13: "How large is the effect of ensiling on maize nutritive value?"
Values for starch and crude protein (CP) were generally greater in ensiled forage compared to fresh forage. Forages with a high cell wall fraction tend to lose more hemicellulose than forages with a small cell wall fraction, leading to a smaller variation in NDF between varieties in the ensiled product. Ensiling most severely influenced NDFD while OMD was least influenced.

RQ14: "What is the optimal harvest date, calculated from analyses of maize silage?"
Ensiling did change the starch concentration and OMD but the harvest date(s) with maximal values for fresh forage also showed maximal values after ensiling. Harvesting the silage at a DM concentration of 32-35% guaranteed an optimal harvest date.

RQ15: "How large is the harvest window, calculated as a set of harvest dates for which the variety ranking of the fresh forage corresponds with the variety ranking at the optimal harvest date calculated from the ensiled forage?"
The harvest window ranged from 2800 to 3100 OU, corresponding with a flexible harvest period of about 21 days. The variety rank based on fresh forage at any harvest date within this range equalled the variety rank based on analyses of ensiled forage at H_{opt}.

Conclusion: *H4 is supported.*
Varieties with a superior fresh nutritive value keep their leading position after ensiling, but variety differences become smaller after ensiling. Reporting variety ranks without going through the ensiling process continues to be a scientifically justified practice in the Belgian Official Variety Trials.

8.1.7 Hypothesis H5: Drought influences the nutritive value of maize

RQ16: "How large is the effect of drought on maize nutritive value during maturation?"
Results on the effect of drought on maize nutritive value were contradictory between years. Drought stress influenced maize nutritive value negatively in 2013, but maize nutritive value improved due to drought stress in 2015.

Conclusion: *H5 is only partly supported.*
A stressed maize plant adapts its stover-ear relation. Being confronted with a drought period does not automatically mean that maize nutritive value is negatively influenced.

8.2 Implications for the Belgian official variety trials

The incentive of the research reported in this PhD manuscript was a long lasting debate among the maize breeding companies and the conductors of the Belgian National List Variety Trials. The points of discussion were: (1) do methods used to measure nutritive value produce relevant and reliable results, (2) does plant type and harvest date interfere with variety ranking of yield and nutritive value of the tested varieties, (3) is there a need to analyse maize silage instead of the current analyses on dried freshly harvested maize.

(1) Currently, the Belgian National List Variety Trials report OMD as a proxy for the maize nutritive value. A high OMD value results from the combination of starch, which is fully digestible, and cell walls, which are partially digestible indicated by NDFD. Starch is also reported but more as an indicator of ear proportion and maturation status. The method to determine NDFD remains difficult and inaccurate. Currently, we do not recommend to report NDFD; although we acknowledge its importance as an energy source for the ruminants.

(2) Maize varieties differ in SG trait and maturation. The delayed senescence of SG varieties resulted in an increased digestibility of the stover. While some breeding companies improve the maize nutritive value by focusing on the SG trait and improving NDFD, other companies focus on a greater ear fraction. The ear fraction increases during maturation, while the cell wall digestibility decreases. Therefore, the nutritive value is affected by the stage of physiological development at harvest. Nevertheless, we could define a harvest window that guarantees a stable variety rank. Based on our results, performing and assessing variety trials with a single harvest date continues to be a scientifically justified practice in Belgium, provided the whole-crop DM concentration is between 28 and 40% with a maximum difference of 7% between all compared varieties at any harvest date.

(3) Current analyses are conducted on dried, freshly harvested maize while the animal is eating maize silage. The composition of the feed changes by the silage process, but, except for NDFD, variety differences become smaller after ensiling. The varieties with the best nutritive value according to the Belgian National List Variety Trials continue to be the best as a silage. Based on our results, reporting variety ranks without going through the ensiling process continues to be a scientifically justified practice.

8.3 Further research

From this study, some new research questions arose that pertain both fundamental and practical aspects. Studying maize types highlighted the need for a synergy between agronomy and physiology. Campos *et al.* (2004) stated: "*physiologists have functioned more as tour guides than as discoverers along the route to greater yields*". This means that physiologists have generally described what has been accomplished by breeding rather than directing the process. For example, the SG trait was introduced in commercial maize varieties by a selection for delayed senescence without understanding the underlying physiology. A prolonged photosynthetic capacity through delayed leaf senescence has contributed to the increased yield potential of new hybrids compared to old ones (Tollenaar, 1991). In our study, the SG trait differed between commercially available varieties, but varieties with a delayed senescence did not yield more. Therefore, the level of SG trait is mainly determined by the choice of the reference varieties. Further research is needed to find the missing link between photosynthesis and DM production. Our results suggested that the SG trait could be quantified by measuring proxies for photosynthetic capacity. Therefore, these proxies can be used to develop a standard method to determine the SG character of commercial varieties.

The nutritive value depends on the partition of vegetative and generative tissue, and can be described by the chemical composition and their digestibilities. We described the chemical composition by measuring CP, starch and NDF. However, photosynthetic energy can be stored in the stover as sugars when the supply of assimilates exceeds the demand by the developing ear. These water-soluble carbohydrates relate to a better plant health and lodging resistance (Thomas & Smart, 1993). Based on a small selection of samples, the water-soluble carbohydrate concentration in the whole-crop was on average 55 g kg^{-1}DM. However, it is unclear how large the variation in carbohydrates is between varieties and it is unclear if this variation between varieties remains consistent during maturation. More research is needed to understand the role of water-soluble carbohydrates in the perspective of the SG trait, drought adaptation and disease/lodging resistance.

We studied the optimal harvest date for silage maize and concluded that a maximal yield, starch and OMD was guaranteed at a DM concentration between 32 and 35%. The number of OU to reach this DM concentration depended on the location and year. Even when farmers can monitor the maize plant development by measuring DM concentration, plants are harvested at a time with potentially sub-optimal performances. Favourable weather conditions can be a challenge in September/October in Belgium. Fortunately, maize silage production is less dependent on the weather conditions than hay or grass silage production. Furthermore, most farmers depend on the schedule of their contractor to harvest silage maize. It is unclear to what extent that the DM concentration of the harvested product varies in practice.

The results in this PhD manuscript can directly be translated into the Belgian official maize variety trials. Plant types and farming practices continuously change in all crops. We have now assessed the variety trials with silage maize but we think that it is highly recommended to do a similar in depth assessment for other crops.

Appendix A: Daily photosynthetic capacity pattern of the reference plant (Chapter 4)

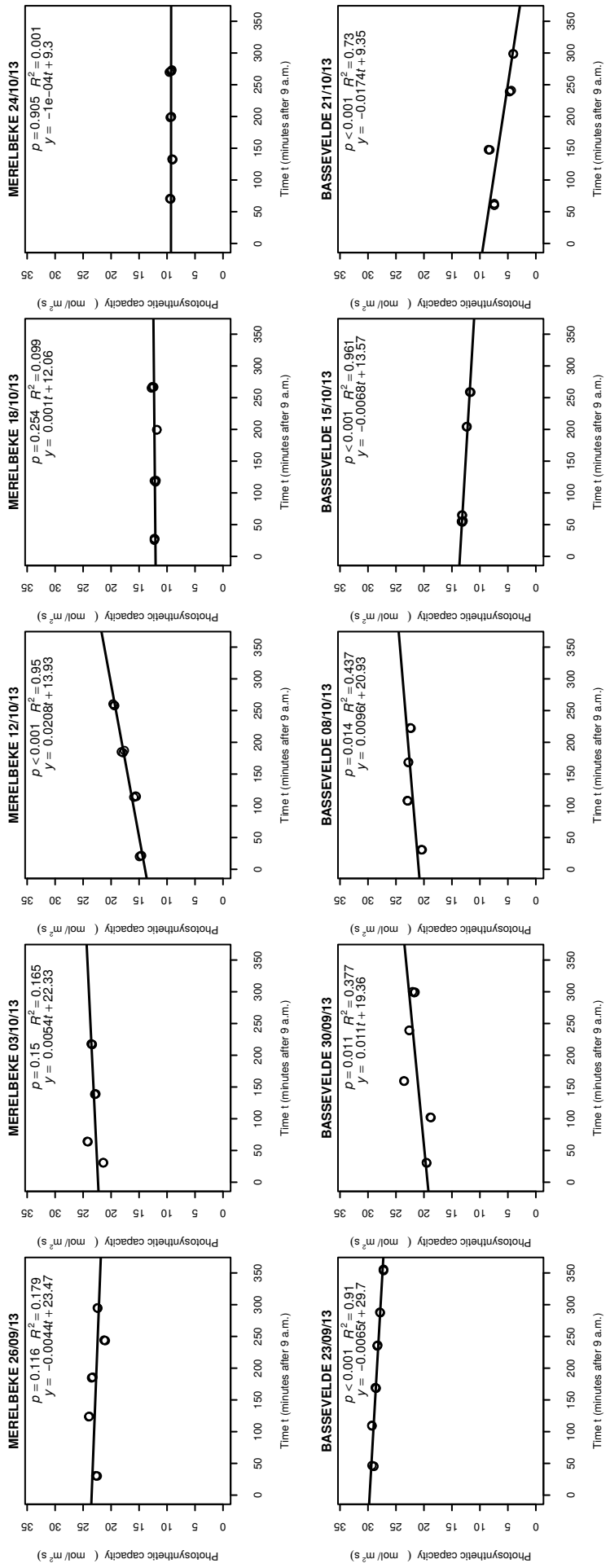


Figure 1: Daily photosynthetic capacity pattern of the reference plant in 2013

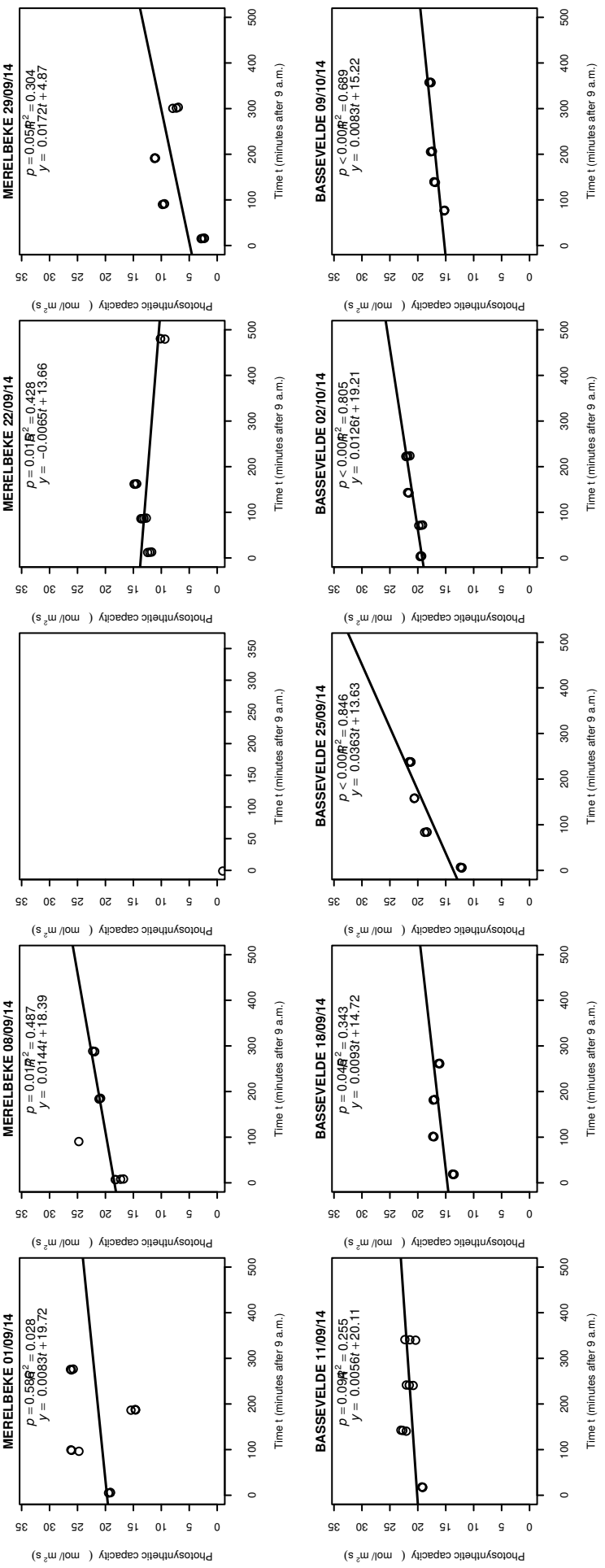


Figure 2: Daily photosynthetic capacity pattern of the reference plant in 2014

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Curriculum vitae

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Scientific publications

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Supervision of MSc thesis students

Wouters, S. (2016) Effect van afrijping en droogte op de kwaliteit van kuilmaïs. Masterproef voorgedragen tot het behalen van de graad master of science in de biowetenschappen: Land- en tuinbouwkunde, KU Leuven technologiecampus Geel.

Capoen, O. (2015) Het effect van inkuilen op de kwaliteit van kuilmaïs. Masterproef voorgedragen tot het behalen van de graad van Master in de Bio-ingenieurswetenschappen: Landbouwkunde, Universiteit Gent.

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